University of Alberta

INFLUENCE OF DIETARY GANGLIOSIDE ON NEONATAL DEVELOPMENT AND GUT PROTECTION BY ALTERING MEMBRANE LIPID PROFILES IN WEANLING RAT

by

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ABBREVIATIONS

AA	arachidonic acid
cAMP	cyclic adenosine monophosphate
Cer	ceramide
CNS	central nervous system
CoA	coenzyme A
COX	cyclooxygenase
CPG	choline phosphoglyceride
EPG	ethanolamine phosphoglyceride
DG	diglyceride
DHA	docosahexaenoic acid
E.coli	*Escherichia coli*
EGF	epidermal growth factor
eNOS	epithelial nitric oxide synthase
Gal	galactose
GLC	gas liquid chromatograpy
GD3	disialoganglioside GD3
GG	ganglioside
Glc	glucose
GLUT	glucose transporter
GM3	monosialoganglioside GM3
GPC	glycero phosphocholine
GPE	glycero phosphoethanolamine
GPI	glycosyl-phosphatidylinositol
I-FABP	intestinal fatty acid binding protein
IL-1β	interleukin-1 beta
IP3	inositol triphosphate
LacCer	lactosylceramide
LCP	long-chain polyunsaturated fatty acid
L-FABP	liver fatty acid binding protein
LOX	lipoxygenase
LPC	lyso-phosphatidylcholine
LPS	lipopolysaccharide
LTB4	leukotriene B4

ABBREVIATIONS (Cont'd)

MUFA	monounsaturated fatty acid
NANA	N-acetyl neuraminic acid (used in thesis text)
NeuAc	N-acetyl neuraminic acid (used in structure)
NF-kB	nuclear factor kappa-B
NOS	nitric oxide synthase
PAF	platelet activating factor
PC	phosphatidylcholine
PDGF	platelet-derived growth factor
PE	phosphatidylethanolamine
PG	prostaglandin
PGE2	prostaglandin E2
PI	phosphatidylinositol
PI-3-K	phosphatidylinositol-3-kinase
PKC	protein kinase C
PLA2	phospholipase A2
PLD	phospholipase D
PUFA	Polyunsaturated fatty acid
PS	phosphatidylserine
SAP	sphingolipid activator protein
SAS	statistical analysis system
SFA	saturated fatty acid
SM	sphingomyelin
Sph	sphingosine
Th	helper T cells
TNF-α	tumor necrosis factor alpha
VEGF	vascular endothelial growth factor

CHAPTER I
INTRODUCTION

1.1. GANGLIOSIDES

1.1.1. Classification of gangliosides, a class of glycosphingolipids

Glycolipids are a structurally diverse group of membrane components found in species ranging from bacteria to man (1). In animal cells, glycolipids can be divided into two major categories, glycoglycerolipids and glycosphingolipids, depending on the nature of the hydrophobic part of these amphiphilic substances. Glycoglycerolipids contain glycerol, fatty acid, or fatty ether and carbohydrate where as glycosphingolipids are composed of sphingosine, fatty acid, and carbohydrate (2).

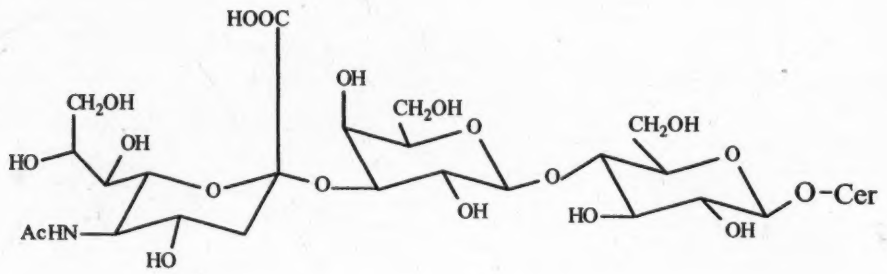
Glycosphingolipids including gangliosides have considerably different chemical structure showing different properties in vivo and in vitro. Glycosphingolipids can be subdivided into 3 groups; neutral glycosphingolipids, sulfato-glycosphingolipids (sulfatide) and gangliosides. Neutral glycosphingolipids consist of a ceramide plus one or more sugars like glucosyl-, galactosyl-, diglycosyl-, or tryglycosylceramide. Sulfatide is classified into acidic glycosphingolipids containing a sulfate ester in galactose. Sulfatide has a similar structure with galactosylceramide except for the sulfate in galactosylceramide. The hydrogen (H) at position 3 of galactose is substituted by sulfate ester (SO^2_4) (3). Sulfatide is thus called as sulfogalactosylceramide (3). Gangliosides are more complex glycosphingolipids which consist of an aliphatic long chain base, a sphingoid connected to fatty acid by an amide bond, and one or more sugars with one or more sialic acids (also called neuraminic acid). Neuraminic acid can be substituted with either acetyl or glycosyl groups (4). These two different types of neuraminic acid are dependent on species (4). For example, only N-acetyl neuraminic acid (NANA) is present in humans but rats have both acetyl and glycolyl neuraminic acids (5).

1.1.2. Nomenclature of gangliosides

There are more than 3000 different structures of sphingolipids (6, 7). Abbreviations for these lipids are recommended by the *Nomemclature Commission of the International Union of Biochemistry* (2) and use three-letter symbols for the sugar plus Cer for ceramide. For example, galactosylceramide is abbreviated GalCer. More complex

glycosphingolipids are separated into a particular family by using a subscript letter to identify the number of sugar units in the chain. *Roman* numeral prefixes indicate the monosaccharide residue counting from the ceramide. A superscript *Arabic* number means the position of the glycosidic linkage. For example, II^3-N-acetylneuraminoslyl-gangliolactosylceramide is II^3-NeuAc-LacCer (GM3) and II^3-N-acetylneuraminoslyl-digangliolactosylceramide is II^3-$(NeuAc)_2$-LacCer (GD3) (Figure I-1) (Table I-1).

(A)



(B)

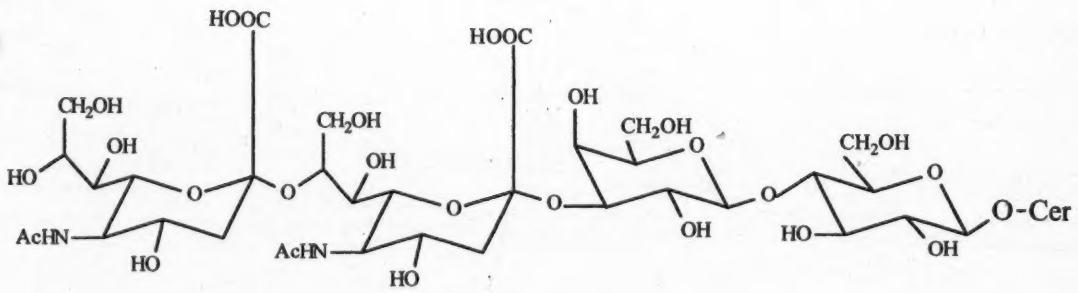


Figure I-1. Structure of ganglioside GM3 (A) and GD3 (B).

Nomenclature by Svennerholm (8) has been commonly used because of it's simplicity (Table I-1). The first capital letter "G" stands for ganglioside. This letter is followed by one of the next *Latin* letter initials: M, D, T, Q and P corresponding to one,

two, three, four and five NANA. Gangliosides are, thus, called monosialoganglioside, disialoganglioside, trisialoganglioside followed by a subscript number 1, 2, 3 meaning a different number of sugar residues in the oligosaccharide moiety (1: four residues, 2: three residues, 3: two residues and 4: one residue) and a, b and c meaning the molecular biosynthesis pathway, respectively (Figure I-2). Therefore, GM3 stands for a monosialoganglioside with two sugars while GD3 is a disialoganglioside with two sugars. GD1b corresponds to a disialoganglioside having four sugar residues different from GD1a synthesized in the a-series pathway. GM3 and GD3 are simpler compared to II^3-NeuAc-LacCer and II^3-$(NeuAc)_2$-LacCer as shown in Table I-1. Many scientists do not use a subscript letter due to convenience. I will follow the general recommendation of Nomenclature by Svennerholm (8) and use a subscript letter.

Table I-1. Nomenclature of Gangliosides

Svennerholm	IUB
GM_4	I^3NeuAc-GalCer
GM_4	I^3NeuAc-GlcCer
GM_3	II^3NeuAc-LacCer
GM_3	II^3NeuGc-LacCer
GD_3	II^3 $(NeuAc)_2$-LacCer
GD_3	II^3 $(NeuGc)_2$-LacCer
GD_3	II^3 (NeuAc, NeuGc)-LacCer
GM_2	II^3NeuAc-$GgOse_3$Cer
GD_2	II^3 $(NeuAc)_2$-$GgOse_3$Cer
GM_1	II^3NeuAc-$GgOse_4$Cer
GD_{1a}	II^3NeuAc IVNeuAc--$GgOse_3$Cer
GD_{1b}	II^3 $(NeuAc)_2$Cer
LD_1	IV^3 $(NeuAc)_2$-nLcOse$_4$Cer
GT_{1a}	IV^3 $(NeuAc)_2$-II^3 NeuAc-$GgOse_4$Cer
GT_{1b}	IV^3NeuAc, II^3 $(NeuAc)_2$-$GgOse_4$Cer
GT_{1c}	$II^3$$(NeuAc)_3$-$GqOse_4$Cer
GQ_{1b}	$IV^3$$(NeuAc)_2$ II^3 $(NeuAc)_2$-$GgOse_4$Cer
GQ_{1c}	IV^3NeuAc II^3 $(NeuAc)_3$-$GgOse_4$Cer
GP_{1b}	IV^3 $(NeuAc)_3$ II^3 $(NeuAc)_2$-$GgOse_4$Cer
GP_{1c}	IV^3 $(NeuAc)_2$ II^3 $(NeuAc)_3$-$GgOse_4$Cer

International Union of Biochemistry (IUB) (9).

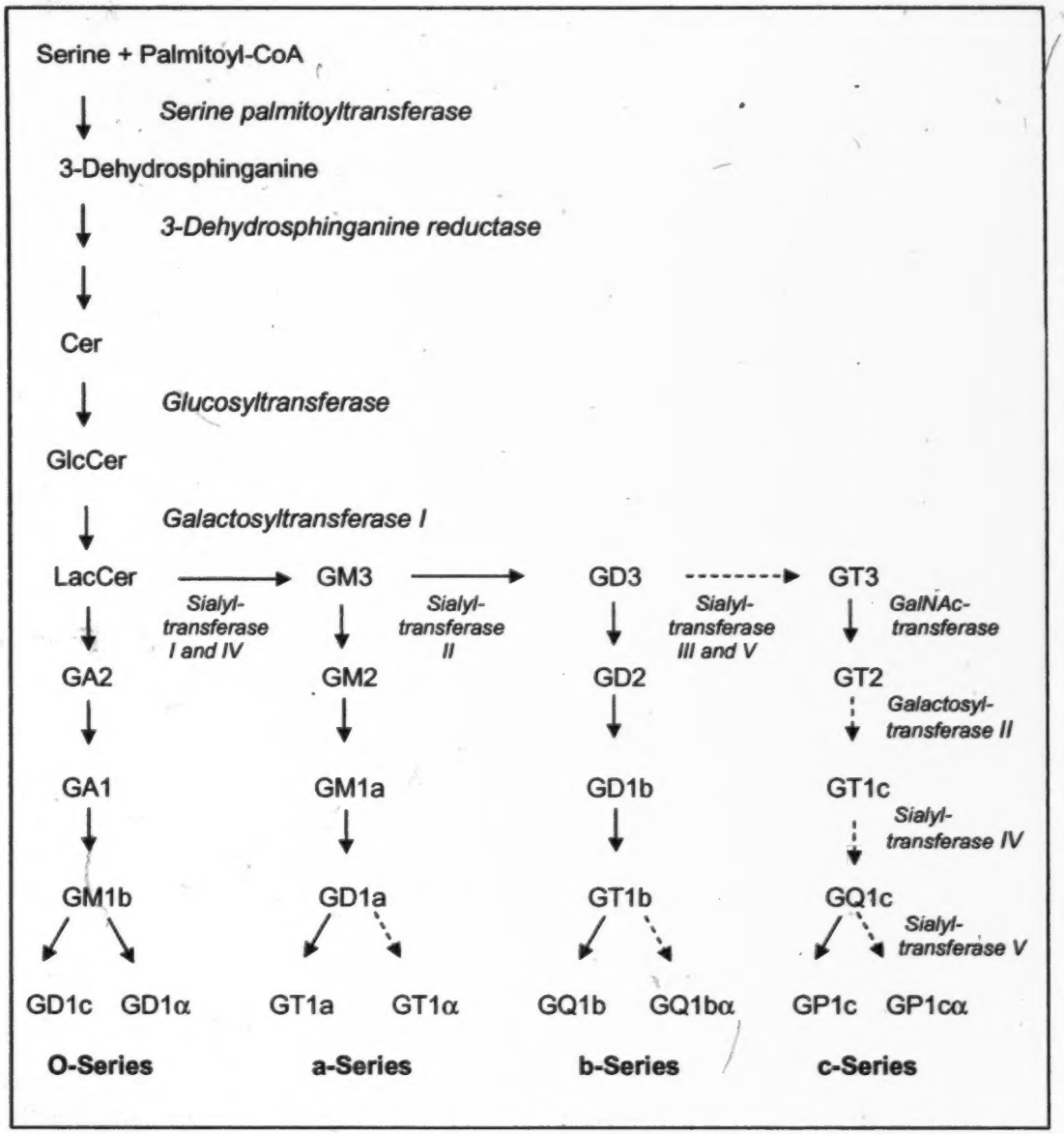


Figure I-2. Ganglioside biosynthesis by the membrane-bound glycosyltransferase in the Golgi apparatus (10).

1.2. DISTRIBUTION AND COMPOSITION OF GANGLIOSIDES

1.2.1. Neural tissues

Gangliosides are predominantly localized in neuronal cell membranes especially in the outer membranes of microsomes and synaptosomes (11, 12) where they constitute

about 10% of total membrane bound lipids in the central nervous system (13). Gangliosides are recognized as a characteristic component in neural tissues such as brain, spinal cord, myelin, and retina (14). Gangliosides are specific to species, tissues, ages as well as sex (15-17). The concentration of gangliosides undergoes considerable changes in the content and pattern during brain development (16, 18). For example, in human brain, ganglioside content increases about 3-fold from the 10th gestational week to the age of about 5 years (18). Gangliosides GM1 and GD1a increase 12~15-fold during the same period with rapid increase occurring around term, during the period for dendrite arborization, outgrowth of axons and synaptogenesis (18). A significant increase in the synthesis of complex gangliosides such as GM1, GD1a, GD1b, and GT1b, takes place during axonogenesis while the synthesis of GD1a and GT1b increases during dendritogenesis, dendrite growth, and synaptogenesis (19). Change in major ganglioside classes also occurs in developing retina (20, 21). At day 8, the major ganglioside GD3 decreases (60% to 27%) while by day 15, GD1a becomes the major ganglioside (2% to 49%) in chick retina (Table I-2) (20). GD3 is predominant in the early developing

Table I-2. Ganglioside content in the retina from different species

	Human[a]	Chicken			Rat		
Retina	Whole	Whole[b]	Neural[a]		Whole[a]	Inner[c]	Outer[c]
Day			day 8	day15			
Total GG[a]	3.8	10			12	5.9	2.4
Total GG (%)						73.5	26.5
Ganglioside (%)							
GM1	6.1	1.6	n.d	1.5	3.6	7	7
GM2	1.1	5.8			2.3		
GM3	9.2	6.2	1.3	2.1	9.2	2	10
GD3	28.9	14.6	60	27	36.5	28	46
GD1a	15.7	45.2	2	49	10.7	16	12
GD1b	14.1	7.5			10.5	23	22
GT1b	14.5	15.0	7.1	15	19.1	20	3
GQ1b	4.6	5.1			4.0	4	
GT3			28	3.1			

[a] Data presented by Daniotti et al (20) as % of total ganglioside NANA (nmole/mg protein). [b] Daniotti et al (22). [c] Dreyfus et al (15) as % and nmol/mg protein of total gangliosides.

stage of bird and mammalian central nervous systems. GD3 is a major ganglioside in mature mammalian retina (20) localized at the photoreceptor in the outer retina (Table I-2) (15, 21). In the outer retina, GD3 consists of about a half of the total gangliosides (15). GD3 is thus a biomarker of retinal development. Together, changes in gangliosides in composition and content in neural tissues suggest that gangliosides are involved in development of neuronal function such as synaptogenesis, neuritogenesis and recovery of injured neuronal tissues (23-29).

1.2.2. Extraneural tissues and fluids (milk and blood)

Extraneural tissues have a lower concentration of gangliosides when compared to the level of gangliosides in neural tissues (Table I-3). The microvillus membrane of animal intestine has exceptionally high amounts of glycosphingolipids including gangliosides relative to other lipids (30, 31). Rat intestine has 20%-30% of the total lipids as glycosphingolipids (31). Thirty four percentage of total glycosphingolipids consist of gangliosides (31). In pig intestinal microvillus membranes, gangliosides are about 7% of the glycosphingolipids which consist of 29% of total phospholipids (30). Total content of glycosphingolipids in microvillus membranes is four times higher compared to their content in mucosa (31). GM3 is the major ganglioside in the rat intestine and is mostly localized at differentiated microvillus cells compared to undifferentiated crypt cells (32). The CMP-sialic acid:lactosylceramide sialyltransferase enzyme, responsible for the sialylation of lactosylceramide to synthesize GM3, is significantly higher in differentiated microvillus cells compared to undifferentiated crypt cells (32). The intestine also has developmental changes in the content and composition of gangliosides. Intestinal GM3 content reaches maximum at 6 days with as much as 315 μg of NANA per g of intestine and quickly falls over 2 week, accounting for 45 μg per g of intestine at 60 days with a compositional change from 80~90% to 72% (33). The content of ganglioside is highest in the distal segment, intermediate in the middle and lowest in the proximal segment in rat intestine with regional variation in the fatty acid composition (34).

There is also ganglioside specificity in structure that depends on species, tissues, cells and fluids. For example, human tissues, except for tumor cells, contains only the N-acetyl neuraminic acid (NeuAc or NANA) form of GM3 not N-glycolyl neuraminic acid

(NeuGc) while NeuGc is detectable in animals (35). In human plasma, NANA is a principal type but 50% of neuraminic acids in gangliosides is NeuGu in bovine plasma (36). This composition changes during development (Table I-4). The NeuGu content in bovine colostrum is high up to 20% of total gangliosides but is only 8% by day 90 of lactation (37). The major ganglioside, in human milk, is GD3 and has a remarkable change during the period of lactation (Figure I-3), from 70% of total ganglioside at day 2-3 to 9% by day 60-390 (38). GM3 increases from 9% of total ganglioside to 84% during lactation (Table I-4). GM3 is only a minor component (3%) of bovine milk ganglioside (39) (Table I-4).

Table I-3. Ganglioside content in extraneural tissues

Tissue	Small intestine		Kidney[c]		Muscles[d]		Mice ICR[e]			
Species	Human[a]	Rat[b]	Rat		Human	Mouse				
	Mucosa	Whole	BBM	BLM			Pregnancy		Lactation	
Day							6	18	2	10
Total GG (µg)		7.8			19.0	7.2				
Ganglioside (%)										
GMI	20		n.d	n.d	-	tr	78	44	21	20
GM2	4				27	tr				
GM3	26	87	76.8	51.6	37	61	217	118	90	57
GM4				22.9	-	-				
GD3	18		9.8	23.8	3	tr				
GD1a	8		3.6	1.7	12	6	57	7	10	8
GD1b	6				1	tr	n.d	1	121	163
GT1b					4	3				

[a] Data presented by Keranen (40) as % based on a molar basis (mole NANA/number of NANA in a ganglioside. [b] Dahiya et al (34) as % of total gangliosides. [c] Spiegel et al (41) as % of total gangliosides. [d] Nakamura et al (42) as % of distribution by a densitometric calculation. Total content was µg/g wet tissue. [e] Momoeda et al (43) as nmol/g dry tissue.

1.3. METABOLISM OF GANGLIOSIDES

1.3.1. Degradation

Glycosphingolipid (GSL) degradation proceeds stepwise with liberation of individual constituents starting from the non reducing terminus of the oligosaccharide in

reverse order to that of biosynthesis (10, 44). This process leads to ceramide that is split by ceramidase into a sphingosine and a fatty acid (45). The degradation of gangliosides from plasma membrane reaches the lysosomal compartment mainly by an endocytic membrane flow through early and late endosomes (46). More than ten different exohydrolases are known to be involved in glycosphingolipid degradation when vesicles reach the lysosome (46). Enzyme deficiency results in accumulation as the corresponding

Table I-4. Ganglioside composition in milk

	Human[a]		Cow[b]
Ganglioside (%)	2 day	390 day	Normal
GM1			
GM2			6
GM3	9	84	3
GD3	70	9	80
GD1a and b			11
GT1a and b			
GQ1b			

[a] Data presented by Takamizawa et al (38) as % of total lipid bound sialic acid.
[b] Laegrid et al (39).

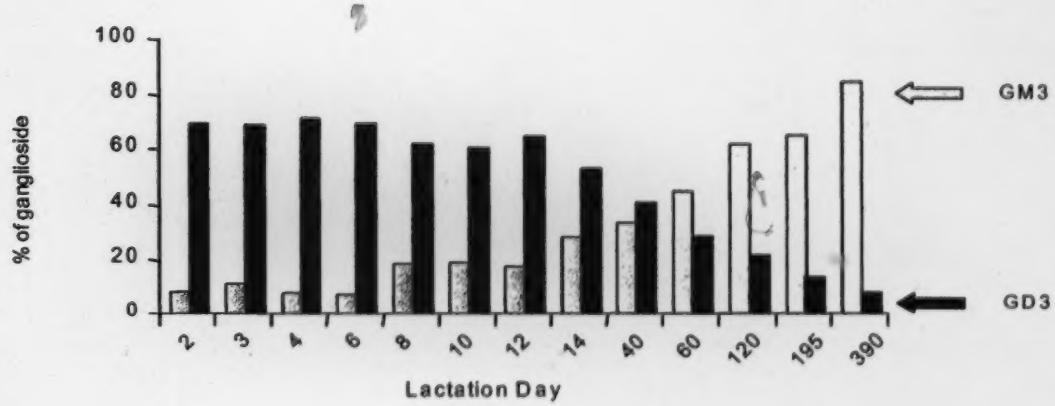


Figure I-3. Developmental changes of GD3 and GM3 in human milk (38).

lipid substrate and is sorted in the lysosomal compartment (46). These undesirable disorders result in corresponding inherited lipid storage diseases (46). Degradation of ganglioside requires a lysosomal ganglioside-binding protein, GM2- activator protein, and β-hexosaminidase A to form water soluble complexes (47). For example, GM2, a ganglioside having a short carbohydrate chain, cannot reach the water soluble enzyme. A second component, called GM2-activator protein, which lifts GM2 from the membrane and presents it to the hexosaminidase A, is required for GM2 degradation (46). Sphingolipid degradation is not necessarily restricted to occur in the lysosome. Sphingomyelin and ceramide can be hydrolyzed by neutral sphingomyelinase (SMase) and neutral ceramidase in the plasma membrane and microsome (48) compared to acid SMase in the lysosome. The brush border membrane at the intestinal mucosa contains high amounts of alkaline SMase to hydrolyze dietary sphingomyelin to ceramide (49). Ceramide is degraded to sphingosine and a fatty acid by intestinal ceramidase (50). Earlier studies show that sphingosine is metabolized to a free fatty acid and incorporated into triglyceride (51). Radiolabelled studies demonstrate the use of sphingosine for phospholipid synthesis, as a donor of hexadecanal for ether phospholipids and ethanolamine- and choline-phosphate for phosphatidylethanolamine and phosphatidyl-choline synthesis, respectively (52, 53). Gangliosides are important for a rapid growth of infant neuronal tissues (16). Ether phospholipids are also known to be necessary for neuronal growth and protection (54). Neonates consume gangliosides and ether phospholipids from milk (55). However, it is not known if dietary gangliosides can alter the synthesis of ether phospholipids during gut development.

1.3.2. Absorption and transport

Glycosphingolipids (GSL) are constituents of most foods such as dairy products, meats, egg, fish, vegetables, fruits and cereals (56). The amount of GSL is relatively small compared to phospholipids, cholesterol and triglyceride (57). Since dietary sphingolipids can be reutilized for phospholipid and triglyceride synthesis in the enterocyte (52, 53), the absorption of dietary sphingolipids may be important for gut physiology and function during development. Absorption of dietary gangliosides is not fully understood, especially during early development. Sphingolipid consumption for

adults is estimated to be approximately 0.3~0.4 g/day (58). The new born human ingests about 400~500 mL milk/day and a 4 month-old baby consumes 550~1000 mL milk/day. Human milk contains sphingomyelin (39~119 mg/L), glucosylceramide plus lactosylceramide (12.5~36 mg/L), and gangliosides (11~16 mg/L) (59), therefore suckling babies thus ingest about ~35~170 mg sphingolipids including sphingomyelin, glycosphingolipids and gangliosides per day.

Several studies show that dietary sphingomyelin and cerebrosides are metabolized by SMase and ceramidase through the stomach, small intestine and colon in rats and mice (49, 60-62). The process of digestion and absorption seems to be slow in the intestine (53). Absorption is poor because sphingomyelin or cerebrosides administered to animals are found in feces as metabolites and in the intact form (25%~43%) (49, 51, 60). Hydrolyzed sphingolipids are transported to lymph (51) and serum (63) and found in the liver (53). These findings suggest that some components of dietary sphingolipids are absorbed and transported into other tissues by the circulation. Chylomicrons are involved in transport of sphingolipids, and lymph contains about 1 nmole/mL of sphingolipids (64). An early study demonstrates high amounts of glycosphingolipids in chylomicron and lipoproteins from human plasma (65). For example, 15~25% of each glycosphingolipid such as glucosylceramide, lactosylceramide, trihexosylceramide and globoside is found in the very low-density lipoprotein and chylomicron fraction (65). Low density lipoprotein and high density lipoprotein consists about 30~45% and 40~50% glycosphingolipids, respectively (65). Although there is not direct information on ganglioside digestion and absorption, several studies indicate that sialidase exists in saliva, gastric, intestinal secretion and mucosa as well as in human milk (66-68). There appears to be a positive correlation between intestinal sialidase activity and the sialic acid content in milk of suckling rats and 12 other species (69). These findings suggest that dietary gangliosides are hydrolyzed by sialidase in the gastrointestinal tract from birth onward.

1.3.3. Biosynthesis

Ganglioside biosynthesis in tissues is coupled to stepwise movement along a membrane pathway from the endoplasmic reticulum to Golgi compartments (44, 70). The

various steps in ganglioside assembly occur on the luminal surface of subsequent *cisternae* of the Golgi complex (71). The end product of biosynthesis is destined for the plasma membrane which contains three-fourths of the total cell gangliosides (72).

De novo biosynthesis of ganglioside starts with condensation of serine and palmitoyl-CoA by the pyridoxal phosphate-dependent serine palmitoyltransferase in the endoplasmic reticulum, making 3-dehydrosphinganine (Figure I-2) (10). This step acts as the rate-limiting step of glycosphingolipid biosynthesis (73). After two more steps, making D-erythro-sphinganine and D-erythro-dihydroceramide, ceramide is made with a fatty acid on the cytosolic surface of the endoplasmic reticulum (74). Ceramide is used as a precursor for sphingomyelin synthesis by phosphatidylcholine: ceramide phosphorylcholine transferase or for glucosylceramide, a primary glycosphingolipid, by ceramide glycosyltransferase (75). Glucosylceramide is the precursor for synthesis of lactosylceramide by galactosyltranferase on the cytosolic side of the Golgi apparatus (trans Golgi/trans Golgi network) (71, 75). The synthesis of galactosylceramide takes place in the lumen of the endoplasmic reticulum (75).

Ganglioside GM3 is a first ganglioside synthesized from lactosylceramide by sialyltransferase in the terminal Golgi apparatus (76). GM3 is a precursor for GM2 or GD3 synthesis, which characterizes the 'a-' and 'b-' series ganglioside pathway (10) (Figure I-2). Synthesis of GD3 occurs in the *cis* Golgi compartment. More complex gangliosides such as GD1a, GT3 and GT1b, are synthesized in the *trans* Golgi compartment and network (77, 78). Gangliosides synthesized in the Golgi apparatus are destined to be transported to the plasma membrane within 20 min (79). A small fraction of ganglioside is known to recycle to the plasma membrane via the Golgi apparatus (80). As serine palmitoyltransferase is the rate-limiting enzyme for synthesis of sphingolipids, both GM2 and GD3 synthases have key regulatory roles in ganglioside synthesis (44).

1.4. FUNCTION OF GANGLIOSIDES

1.4.1. Biological and physiological function

1.4.1.1. Cell adhesion and recognition

Glycosphingolipids, especially gangliosides, are known to have a role in cell adhesion and cell recognition (81). There are three different modes by which gangliosides

bind to the cell surface: loosely associated micelles removable by serum, tightly attached micelles removable by proteases such as trypsin and ganglioside molecules inserted into the outer leaflet of the plasma membrane (81). Binding is based on carbohydrate-carbohydrate interaction (82). For example, mouse B16 melanoma cells display high content of GM3 and adhere to lactosyl ceramide, globotriosyl ceramide, or globotetraosyl ceramide coated plates but cell adhesion is suppressed by treatment with anti-GM3 monoclonal antibody DH2 or by sialidase (83). The cell adhesion process is rapid, specific and dependent on bivalent cations such as Ca^{2+} (82). Glycosphingolipids bind cells by myeloglycan, a major physiological epitope in E-selectin-dependent binding to polylactosaminolipids of leukocytes and HL60 cells (84, 85). Gangliosides also act as modulators for integrin receptor function (82). For example, GT1 and GD1a prevent attachment of cells to the fibronectin-collagen complex (86). GD2 increases vitronectin receptor activity (87). GM3 enhances fibronectin receptor activity (88) .

1.4.1.2. Cell differentiation and proliferation (neuritogenesis & synaptogenesis)

The attempts to elucidate glycosphingolipid function have focused on brain neurons. Gangliosides are known to play important roles in neurobiological functions including neurodifferentiation, neuritogenesis, and synaptogenesis (89, 90). The content and pattern of gangliosides affect neuronal and glial cell differentiation (16, 22, 43, 91). Total ganglioside content increases up to completion of differentiation (91) and is changed during development (16, 22, 43). Most gangliosides, except GM4, have an effect of induction of neuritogenesis (92). Neutral glycosphingolipids without NANA have no activity in neuritogenesis (92). GD3 is considered as a marker for cell division and migration (27). GQ1b is characteristic of nerve cell sprouting and arborization (27). In brain cortex, GD1a seems to be a marker for synaptogenesis and GM1 or GM4 is characteristic of myelination (27).

GM3 influences cell differentiation of astrocytes to oligodendrocytes. This effect is specific for GM3 but not for GM1, GM2, GD3, GD1a or lactosyl ceramide when tested in cell cultures (93). When neural retina cells emit neurites, GD3 is transiently coexpressed, and then GD3 remains as the major ganglioside in differentiated neurons (94). HL-60 cells incubated with GM3 in serum-free medium exhibit a significant

increase in phagocytic and nonspecific esterase activities but also inhibition of cell growth with cell differentiation (95). GM1 activates neuronal differentiation by facilitating Ca^{2+} efflux, which reduces the nuclear Ca^{2+} that characterizes the differentiated neuron (96). This suggests that GM1 has a role regulating Ca^{2+} flux across the nuclear membrane during neuronal development. Taken together, the effect of gangliosides on cell differentiation and proliferation, including neuritogenesis and synaptogenesis is very dependent on tissue or cell type.

1.4.1.3. Modulation of Ion channels, Ca^{2+} influx and neurotransmitters

Gangliosides contribute in several ways to modulate complex ion channels, transport/exchange proteins, Ca^{2+}-utilizing enzymes and neurotransmitters. The effect of gangliosides on neuritogenesis is via Ca^{2+} influx (97, 98). GM1 modulates Ca^{2+} influx. Plasma membrane-linked Na^{+} and Ca^{2+} pumps and channels appear to be modulated by gangliosides, particularly by GM1 (99). GM1 and GM2 elevate cytosolic-free Ca^{2+} concentration but, GM3 decreases Ca^{2+} concentration during platelet-derived growth factor (PDGF)-induced vascular smooth muscle cell growth (99). Binding of cholera toxin to GM1 on the human Jurkat T cell increases intracellular free Ca^{2+} concentration through both the release of Ca^{2+} from intracellular stores and a Ca^{2+} influx from extracellular spaces (100). GD3 and GM3 induce a rapid, reversible elevation of free cytoplasmic Ca^{2+} in human blood platelets (101). The Ca^{2+}-mobilizing effects of gangliosides in human blood platelets are stimulated by epinephrine but inhibited by substances inducing activation of protein kinase C (PKC) and cAMP-dependent protein kinases (101). GM1 also activates the Ca^{2+}/calmodulin-dependent kinase and enhances spontaneous excitatory postsynaptic currents (102). GM1 activates a sodium-calcium exchanger in the nuclear envelope of NG108-15 neuroblastoma cells (103). Na^{+}/K^{+}-ATPase activity is activated by brain gangliosides, GM1, GD1a, GD1b and GT1b by 26-43% (104).

Exogenous gangliosides also influence neurotransmitter release through facilitation of depolarization induced Ca^{2+} influx into isolated synaptosomes (105). GM1 and GQ1b gangliosides increase acetylcholine (Ach) release by modulating voltage-dependent calcium channels in the synaptic plasma membrane (105). When rat brain slices are

treated with GM1 antibody, depolarization-induced gamma-aminobutyric acid (GABA)-release is markedly enhanced (106). Systemic administration of GM1 increases striatal dopamine levels in the mouse MPTP model of Parkinsonism (107).

1.4.1.4. Immune function

The immune system relies on signaling pathways for activation and amplification for antigen-specific T and B cell clones to combat infectious agents or tumors (108). Potentially harmful auto-reactive T cells are eliminated via apoptosis during their maturation process (108). In the immune system, GD3 stimulates T-cell proliferation in human peripheral blood by tyrosine phosphorylation (109, 110). GD3 induces phospholipase C-γ phosphorylation, calcium flux, Ras and IP3 activation upon cellular response (109). GM3 and GD3 present in elevated amounts in sera of tumor-bearing hosts, inhibit the cytotoxicity of natural killer cells and phytohemagglutinin-induced blast transformation (111). GM3 and GD3 stimulate T-suppressor activity of peripheral blood lymphocytes (111). When T cells derived from peripheral blood are treated with monoclonal antibody (mAb) against GD3, T-lymphocytes proliferate, suggesting that binding to GD3 on the surface of T cells induces signals for T cell proliferation (112).

Ceramide is also involved in immune response. Ceramide modulates ceramide activated protein kinase (CAPK) and mitogen activated protein kinase (MAPK), PLA2, ceramide activated protein phosphatase (CAPP) and NF-kappa B activation (108), significantly involving signals for growth arrest, apoptosis and inflammation (113). Fas, a widly distributed surface receptor belonging to the TNF receptor family, is expressed on all intestinal lamina propria T lymphocytes (114). Fas cross-linking induces sphingomyelin hydrolysis by acidic SMase to ceramide and diglyceride. Ceramide then activates a number of kinases and phosphatases resulting in apoptotic cell death in intestinal lamina propria T lymphocytes, but not in peripheral blood T lymphocytes. It is assumed that the interaction of Fas and Fas ligand may act in the homeostasis of gut mucosa-associated lymphoid compartment.

This immune system response is questionable in the neonatal infant during 1-2 weeks after birth because they don't have immumoglobulin A (IgA) and immumoglobulin (IgM)-producing immunocytes in the intestine during this period (115).

There may be a compensatory relationship between colostrum containing high amounts of ganglioside and the neonatal infant having no secretary immunocytes during the postnatal period. Studies show that dietary gangliosides increase immune response and protection. Dietary gangliosides in weanling mice stimulate maturity of cytokine-secreting cells and increase the number of Th1 and Th2 cytokine-secreting lymphocytes in lamina propria and Peyer's patches lymphocytes (116). Feeding ganglioside-supplemented infant formula to preterm newborn infants show lower content of *Escherichia coli* (*E.coli*) but higher content of *Bifidobacteria* in their feces by 30 days postnatal age compared to neonates fed standard formula (117). Although it is known that gangliosides influence immune function, little is known of whether dietary gangliosides inhibit inflammatory or infective mediators induced by toxins in infected intestine during early development.

1.4.1.5. Signal transduction

Gangliosides transduce signals stimulating cell growth, arrest or influence the function of receptors, transducers and transporters (118). Incubating purified human platelet-rich plasma with ganglioside GM1 and GM3 inhibits cell growth and PDGF-dependent DNA synthesis in 3T3 cells (118). In contrast, GM3 reduces epidermal growth factor (EGF) receptor phosphorylation when incubated with human plasma (118). Increasing EGF receptor auto-phosphorylation and EGF- stimulated cellular proliferation are associated with decreased levels of ganglioside expression in mutant Chinese hamster ovary cells that possess a reversible defect in biosynthesis of gangliosides (119). Similarly, KB cell and A431 cell growth can be arrested by GM3 due to inhibition of EGF-stimulated phosphorylation of the EGF receptor (120). GD3 is known to stimulate production of vascular endothelial growth factor (VEGF), an angiogenic factor, from human glioma cells (121). Nude mice with genetically suppressed GD3 synthase show minimal angiogenesis by down regulating of VEGF (122). GD3 also induces apoptosis resulting from CD95 initiation or ceramide accumulation (123). A higher GD3/GM3 ratio increases new vasculaization (124).

Insulin-stimulated phosphorylation of the insulin receptor (IR) and IR substrate-I is significantly reduced in muscle of transgenic mice with accumulation of GM1 and GM2

due to a defect in sialidase (125). Activities of phosphatidylinositol-3-kinase (PI-3-K) and glycogen synthase are low compared with the wild type (125). Gangliosidosis is a rapidly progressive neurodegenerative disease resulting from accumulation of gangliosides because of defective activity of the lysosomal proteins responsible for ganglioside degradation (126). In a mouse model of Sandhoff disease (Hexb-/- mice), a GM2 gangliosidosis, microsomes from Hexb-/- mouse brain exhibit a significant reduction in Ca^{2+}-uptake via the sarco/endoplasmic reticulum Ca^{2+}-ATPase (126). It is reversely prevented by feeding Hexb-/- mice with N-butyldeoxynojirimycin, an inhibitor of glycosphingolipid synthesis that reduces GM2 storage (126). Insulin resistance induced by TNF-α in 3T3-L1 adipocytes accompanies a concomitant increase in GM3 (127). Depletion of GM3 by an inhibitor of glucosylceramide synthase prevents the TNF-α - induced signaling defect in insulin-dependent tyrosine phosphorylation of insulin receptor substrate-1 (127).

Ceramide also modulates biological functions. Ceramide enhances formation of minor neuronal processes from lamellipodia, a prerequisite for cell spreading and migration, and the subsequent stage of axonogenesis (128). Ceramide induces the arrest of cell growth (129). Exogenous C6-ceramide dephosphorylates Rb (a retinoblastoma gene product) leading to induction of G0/G1 cell cycle arrest in a concentration- and time-dependent manner. Arrest of cell growth is protected by sequestering Rb (129). C_6-ceramide down-regulates transduction of the insulin-responsive glucose transporter gene (GLUT4) by ~60% in 3T3-L1 adipocytes (130). C_2-ceramide and sphingosine stimulate interleukin (IL)-1-mediated prostaglandin E2 (PGE2) which acts as a vasodilator (131). Ceramide inhibits phospholipase D (PLD) activity and then leads to a decrease in phosphatidic acid, a positive regulator of cell growth while sphingosine and sphingosine-1-phosphate stimulate the activity (132). Sphingosylphosphocholine (SPC), generally known as lyso-sphingomyelin, has greater mitogenicity than sphingosine and sphingosine-1-phosphate in Swiss 3T3 fibroblasts (133). Unlike sphingosine and sphingosine-1-phosphate, SPC does not induce intracellular signaling events involving activation of PLD or inhibition of cAMP production (133).

1.4.1.6. Microdomains (Caveolae/lipid rafts) as a platform for sorting, clustering and trafficking of signal transduction events and pathogen entry

Microdomains (caveolae and lipid rafts) are heterogeneously distributed in plasma membranes as platforms to concentrate signaling molecules or any other molecules with the same destination in the cell (134). Caveolae are 50~70 nm plasma membrane pits and has been known as flask-shaped membrane, omega-shaped membrane, or plasmalemmal vehicles because they appear to shuttle molecules across the cell (135). Caveolae are comprised of three different isotypes of caveolin in mammalian cells. Caveolin-1 and -2 are coexpressed in many cells with particularly high levels in adipocytes and form a heterooligomeric complex in many cell types (136). Caveolin-3 is expressed in all classes of muscles (137). In contrast to caveolae, lipid rafts (non-caveolar glycosphingolipid-enriched domains) range from 70 nm to 1 μm (138, 139) and are planar regions of the membrane enriched with signaling lipids and proteins (11). Lipids recovered from microdomains have higher melting points than the average lipids from the plasma membrane suggesting a liquid-ordered phase with highly saturated fatty acids in microdomains (140).

Microdomains are regions of the plasma membrane categorized by their physical structure and chemical composition. Examples of microdomains include caveolae, lipid rafts or glycosphingolipid-enriched signaling domains (135). A recent study demonstrates that lipid rafts are capable of invaginating and becoming caveolae by cellular signaling (135, 141). Epithelial cells treated with α2β1 integrin antibodies induce membrane clustering and clustering starts to move laterally on the cell surface along actin filaments (141). After induction of lateral redistribution of integrin from lipid rafts to caveolae, integrin internalizes into caveosome-like perinuclear structures in a PKC dependent sequence (141) thus suggesting that microdomains are involved in endocytic trafficking mechanisms via caveolae.

Functionally, microdomains have important roles in signal transduction events and the entry of pathogens. Signaling molecules including lipids and proteins are enriched in microdomains (reviewed in (135, 142)). For example, gangliosides, sphingomyelin, ceramide, diglyceride and cholesterol are found in microdomains (135, 142). Acylated- (heterotrimeric Gα and Gβ and caveolin), prenylated- (Ras), and GPI-anchored proteins

(alkaline phosphate) are enriched (135, 142). As membrane receptors, PDGF, insulin, EGF, cholecystokinin, bradykinin, and tyrosine kinase are localized in microdomains (135, 142). Signal transducer, PKC, PLC, MAP-kinase, adenylyl cyclase, PI3-kinase, calmodulin, polyphosphoinositide phosphatase and nitric oxide synthase (NOS) are colocalized in microdomains. Ca^{2+}- and H^{+}-ATPase are also found in these domains associated with membrane transporters (135, 142).

There are several studies showing a primary role of caveolin upon binding or releasing some proteins. For example, the association of epithelial NOS (eNOS) with caveolin in endothelial cells remarkably attenuates enzyme activity (143). Enzyme activity is restored by dissociation of the eNOS-caveolin complex by increasing the Ca^{2+} concentration (143). As Ca^{2+} concentration returns to basal levels, eNOS re-associates with caveolin again (143). Binding of caveolin to insulin receptor stimulates tyrosine phosphorylation of caveolin (144). The β2-adrenergic receptor regulating the intrinsic contraction rate in neonatal mouse myocytes seems to be associated with caveolin-3 (145). Caveolin facilitates the integrin ligation to the tyrosine kinase Fyn for cell cycle sequence of signaling events (146). Thus, increased concentration of proteins or ordered lipid environment in lipid rafts may affect intermolecular interaction or functional properties of receptors (147, 148).

Membrane microdomains have a critical role in entry of different pathogens. Microdomain-localized receptors are critical for entry of different pathogens such as *E.coli*, *cholera* toxin, *Human immunodeficiency virus (HIV)*, *Shiga* toxin, malaria parasite *Plasmodium falciparum*, *Campylobacter jejuni*, *Siman virus 40*, *Chlamydia trachomatis* and *Respiratory syncytial virus* (149, 150). Transmembrane proteins of influenza virus hemagglutinin and neuraminidase induce signals for their association with rafts (151). Shiga toxins produced by *Shigella dysenteriae* and enterohemorrhageic *E.coli* bind specifically to globotriaosylceramide (Gb3) enriched in microdomains (152). *E.coli*-heat labile toxin type I binds GM1 in microdomains to function in signal transduction while GD1a renders the toxin inactive (153). These studies suggest that glycosphingolipids including gangliosides have a particular function affecting the entry of bacterial and viral toxins into the cell.

Accumulating evidence suggests that microdomains are involved in signal transduction and pathogen entry. The importance of cholesterol in microdomain function should be considered. It is known that cholesterol binds caveolin directly (154) and regulates caveolin expression (155). Cholesterol is an important lipid component in microdomains. Therefore, a change in cholesterol content affects membrane trafficking and signal transduction, leading to alteration of cell behavior and entry of pathogens (156). Depletion of cholesterol in microdomains significantly reduced amount of several key protein components of the MAP kinase complex, including Ras, Grb2, Erk2, and Src in quiescent cells (156). These results suggest that the MAP kinase pathway is dependent on the cholesterol level in microdomains for control of cell division (156). Endothelial cells treated with methyl-beta-cyclodextrin, a specific cholesterol-binding agent, release substantial quantities of GPI-anchored Thy-1, glycosphingolipid GM1, CD45, intracellular Lck and Fyn kinases (157). Disruption of microdomains by cholesterol depletion blocks downstream signaling of insulin-like growth factor-1 receptor during 3T3-L1 preadipocyte differentiation induction (158). Human ganglioside-specific sialidase Neu3 localized in microdomains is closely associated with caveolin, but is dissociated by cholesterol depletion (159). These results strongly suggest that cholesterol content in microdomains controls cell signal transduction.

Studies show that cholesterol is also important in cellular entry of many pathogens (149, 160, 161). *E.coli* binds to the macrophage through FimH (a mannose-binding fimbral lectin) for bacterial adherence and colonization of mucosal surfaces (162). The internalization of FimH and *E.coli* complex into cells is blocked by sterol binding or caveolae-disrupting agents such as filipin, nystatin and methyl-β-cyclodextrin (160, 161). The B-subunit of cholera toxin binds GM1 in microdomains to enter the cell (163). The complex is transported to the Golgi apparatus and to the endoplasmic reticulum where the A-subunit is activated (163). The toxin is then delivered to the basolateral membrane to activate adenyl cyclase (164). This cascade overall induces cellular secretion of Cl^- ions and activation of cAMP (164). However, cholesterol depletion by filipin inhibits signal activation by cholera toxin due to disruption of caveolae and caveolae-like structures (165). Disruption of microdomains by nystatin blocks the production of LPS-induced TNF-α (166). *Chlamydia trachomatis*, *Campylobacter jejuni*, respiratory *syncytial virus*,

and HIV-1 are not able to enter cells by a treatment of cholesterol depletion (149). There is no study demonstrating if a diet-induced cholesterol reduction in the intestine inhibits entry of pathogens or inflammatory signals induced by toxins.

Taken together, glycosphingolipids and cholesterol appear to be important lipids involved in physiological and biological functions controlling cell growth, arrest of cell growth, apoptosis and pathogen entry by modulating cell receptors, lipid messengers, ion pump/cannels, neurotransmitters and de novo lipid synthesis in the cell.

1.4.2. Pathological and pharmacological function

1.4.2.1. Inherited diseases

Glycosphingolipids are degraded in the lysosome by exohydrolase and a cofactor called sphingolipid activator proteins (SAP) (10). Long carbohydrate chains more than four sugar residues are efficiently degraded by exohydrolase (10, 47). Glycosphingolipids with short sugars require exohydrolase and SAP (10, 47). For example, gangliosides having short carbohydrates can not reach the water soluble enzyme (46). A second component called SAP is needed, which lifts ganglioside from the membrane and presents it to the hexosaminidase A for degradation (46). There are five SAP proteins such as the GM2-activator, SAP-A to -D (also called saposins) (10, 46). Inherited disorders of sphingolipid degradation are associated with lysosomal storage diseases, such as sphingolipidosis (10, 167). These diseases, expressed in brain in early childhood and result in death within the first year of birth, are a commonest cause of pediatric neurodegeneration disease (incidence: 1/18,000) (10, 168). In GM1 gangliosidosis, the total ganglioside content in gray matter increases 3~5 fold and GM1 comprises about 80% of the total (169). This accumulation results in about 12~20 fold over the control (169). These diseases are from the storage of GlcCer-based glycosphingolipids and deficiencies of the specific enzymes and SAP (Table I-5) (10, 167). In contrast to glycerolipid metabolism, defects in every step in sphingolipid degradation cause inherited diseases. Pathogenesis of these inherited diseases is still poorly understood. Many animal models are recently available to study target genes disrupting sphingolipid metabolism (10).

1.4.2.2. Restoration of neuronal tissues

Gangliosides are naturally abundant in neuronal tissues (8). In neuronal injuries such as hypoxic brain, the level of ganglioside is low and correlates with the degree of CNS impairment (170). The content of brain neurotransmitters, glutamate, dopamine and serotonin is significantly increased in hypoxic animal brains (171). Animals treated with GM1 before the exposure of hypoxia prevent all neurochemical changes in the hypoxic neonatal brain (171). Pretreatment with gangliosides in rat cerebellar granule cells completely blocks neurotoxicity (GT1b > GD1b > GM1) elicited by glutamate and kainate (172). Calcium-ganglioside interactions act as modulators of neuronal function and synaptic transmission (173). Glutamate-induced delayed neurotoxicity is due to sustained increase in intracellular Ca^{2+} concentration (174) which leads to cell damage and death through activation of enzymes such as lipases, proteases and endonucleases (175). GM1 prevents the protracted increase in Ca^{2+} concentration (174). GM1-injected animals having global ischemia show just small loss of Na^{+}/K^{+}- ATPase (15 %), and mitochondrial Mg^{2+}-ATPase (8%) compared to significant loss in saline control (38% and 36%, respectively) (176). Because these enzymes are important for Na^{+}-efflux, K^{+}-

Table I-5. Inherited diseases resulted from the storage disease and enzyme deficiency in sphingolipid metabolism

Disease	Enzyme defect	Sphingolipid stored
Gaucher types 1,2 and 3	β-glucocerebrosidase	Glucosylceramide
Fabry	α-galactosidase	Globotriaosylceramide
Tay-Sachs	Hexosaminidase A	GM2 ganglioside
Sandhoff	Hexosaminidase A and B	GM2 ganglioside
GM1 gangliosidosis	β-galactosidase	GM1 ganglioside
Krabbe	Galactocerebrosidase	Galactosylceramide
Farber	Acid ceramidase	Ceramide
Niemann-Pick	Sphingomyelinase	Sphingomyelin
Globoid cell leukodystrophy	Psycosine galactosidase	Ganglactosylsphingosine
Sialidosis	Neuraminidase	GM3 ganglioside

Summarized from reviews (10, 167).

influx and ATP synthesis, decrease in activity of these enzymes causes an energy-dependent ionic imbalance in the plasma membrane and a loss of mitochondrial function (176). GM1 reduces infarct volume after focal cerebral ischemia (177) and results in recovery of retinal layer thickness caused by ischemic damage (178). Administration of gangliosides almost completely results in recovery of marked reduction in Na^+/K^+-ATPase in sciatic nerves in streptozocin-induced diabetic rats (179). These results suggest that gangliosides are protective of neuronal damage.

1.4.2.3. Anti-colon carcinogenesis

Several studies demonstrate that dietary sphingolipids have anti-colon carcinogenic effect (180, 181). These observations may result from multiple effects of sphingolipids in modulating cell growth and apoptosis through PKC (182), PI3-kinase (183), other protein kinases (184), and phosphoprotein phosphatases (185). Mice fed 0.025% (w/w) sphingomyelin in a diet show a 20% incidence of colon tumors compared to 47% in control animals (181). In this study, 0.025% (w/w) GM1 inhibits colonic aberrant crypt foci, an early stage of colon carcinogenesis (181). GluCer, LacCer and GD3 (0.025 or 0.1 g/100g) also reduce colonic cell proliferation and aberrant crypt foci, especially in the upper half of crypts, compared to control animals in 1,2-dimethylhydrazine treated CF1 mice for 4 weeks (186). C57Bl/6J (Min/+) mice fed AIN 76A diet with complex sphingolipids which mimic the composition of milk fat sphingolipids reduce total number of tumor cells by 40% compared to the control diet without sphingolipids (187). Since complex glycosphingolipids are hydrolyzed by digestion along with the gastrointestinal tract (60, 61, 81), this study suggests that derivatives from complex glycosphingolipids are involved in anti-colon carcinogenesis (188). Ceramide alone, particularly long chain ceramide, reduces cancer cell growth by 40% compared to animals fed a control diet without sphingolipids (187). In human colon cancer cell lines, sphingosine, a derivative of sphingolipid, is cytotoxic and decreases total tumor cell number by 50% after 24 h incubation (187). These results suggest that dietary sphingolipids including gangliosides inhibit colon carcinogenesis.

1.4.2.4. Anti-inflammation

Sphingosine and sphingomyelin inhibits PKC and PLA2 activity, respectively, both of which are generally increased in inflammation (182, 189). There are several studies showing anti-inflammatory effects of gangliosides in certain tissues and cells. A natural mixture of gangliosides reduces inflammation and pain in the edemous rat paw developed 1 h after carrageenin injection (190). A mixture of brain gangliosides (10~40 μg/paw) inhibits rat hind paw edema induced by PLA2 (5 μg/ paw) or 1% carrageenin injection. GM1 inhibits arachidonic acid release when stimulated with maitotoxin or melittin in bovine aortic endothelial cells, suggesting PLA2 inhibition by GM1 (191). GM1 reduces the EtOH-induced activation of arachidonyl-specific PLA2 activity in human neuroblastoma cells (192). Gangliosides inhibit plasmalogen-selective PLA2 (193). NANA derived from ganglioside by sialidase decrease PLA2 activity (194). Gangliosides GM3, GD3 and GT1b also inhibit PKC activity and endogenous protein phosphorylation by PKC (195). Gangliosides decrease PLC activity (196), resulting in low level of diglyceride which activates PKC (182). Gangliosides from Italian buffalo milk and its serum decrease PG series 2 production by 75~80% in cultured human colonic epithelial cells exposed to TNF-α (197). In neuronal death after ventral root avulsion, inflammation develops in the wounded area resulting in additional neuronal loss as well as the degeneration of the ventral and lateral funiculi fibers. GM1 enhance surviving motor neurons and decrease local inflammation by reducing the number of degenerating fibers (198). Taken together, dietary sphingolipids have anti-inflammatory effects in tissue and cell specificity.

1.5. FACTORS AFFECTING GANGLIOSIDE METABOLISM

1.5.1. Age

It is important to note that the content and composition of gangliosides dramatically changes during development. Total content of gangliosides in neonatal rat cerebrum is about 0.1 μmole per brain at birth but rapidly rises up to 1.4 μmole at 20 days old (16). The major ganglioside at birth is GD1a (about 48%) (16). During aging, GD1a slowly decreases up to 25% while GM1 becomes the major ganglioside by 1 year of age in human frontal brain cortex (16). The intestine has a relatively high content of

gangliosides from birth to the age of 2 weeks compared to the ganglioside content of the intestine at 60 days of age (33). During this early developmental period of the infant, the ganglioside content of intestine corresponds to the higher content of the mother's milk (199). These data suggest that gangliosides in milk may be an important source of intestinal gangliosides for protection from pathogens during early development.

1.5.2. Dietary nutrients

Dietary nutrients, especially fats, can significantly affect de novo synthesis of sphingolipids including glycosphingolipids and gangliosides during development. Animals fed low (1.2%) dietary fat exhibit a third level of glycolipids in the epiphyseal cartilage compared to animals fed high dietary fat (10.5%) (200). More importantly, maternal essential fatty acid deficiency affects glycolipid metabolism in the fetus (201). When rats were fed diets providing 0.7% calories from essential fatty acids (linoleate + linolenate) 10 days before mating, the subsequent content of glycolipids in the pup's liver is significantly decreased by about 20%. This glycolipid reduction occurs at both day 15 and day 20 of pregnancy compared to animals fed the diet providing 4.8% of calories from essential fatty acids (201). Oral administration of NANA into rat pups for 8 days increases the content of gangliosides in the cerebrum and cerebellum (202). Only a small portion of NANA is absorbed by the intestine and is transported to the brain (203). Animals under malnutrition exhibit a very low and slow rate of the dietary NANA incorporation into brain gangliosides compared to well-fed animals (204). These results suggest that the de novo synthesis of gangliosides in developing animals is significantly altered by the maternal and early infant nutrients. Furthermore, vitamin B_6-deficient rats express impaired enzymatic synthesis of 3-ketodihydrosphingosine in vitro, while addition of vitamin B_6 cofactor induces restoration of this enzyme activity (205). Vitamin D_3 stimulates sphingomyelinase in human pro-myelocytic leukemia HL60 cells leading to increase of ceramide and phosphorylcholine (206). Since there is no study showing how dietary gangliosides affect the lipid profile in early brain development, it would be of interest to determine if dietary gangliosides alter the content and composition of gangliosides in brain through intestinal absorption.

1.5.3. Toxins

Fumonisin produced by *Fusarium moniliforme* is known to alter sphingolipid metabolism (207). This toxin inhibits sphinganine *N*-acyltransferase that is involved in de novo synthesis of sphingolipids by disrupting the acylation of a fatty acid to sphinganine to form dihydroceramide (207). To date, fumonisin FB1, B2, B3, B4, C1, *Alternaria Alternata f. sp. Lycopersici* host-specific toxins (AAL toxins) and *australifungins* are known to inhibit ceramide synthase (207, 208). These toxins are related fungi that are prevalent on corn, sorghum, millet, and other agricultural products (207). In animal studies, low dose of fumonisin appears as tumor promoters (209). Fumonisin B1 increases intracellular free sphingoid base, inhibits phorbol dibutyrate binding and reduces the amount of membrane-associated PKC (210). Fumonisin B1 decreases axonal growth with a decrease in complex sphingolipid synthesis in hippocampal neurons (211). Myrosin inhibits ceramide synthesis by disrupting serine palmitoyltransferase at the de novo rate limiting step (212). Inhibition of ceramide synthesis results in a rapid and specific reduction in the rate of transport of glycosylphosphatidylinositol (GPI)-anchored proteins to the Golgi apparatus (212). Fumonisin B1 also almost completely blocks the folate receptor-mediated uptake of 5-methyltetrahydrofolate by reducing sphingolipids in cell membranes by 40% (213). These studies suggest an importance of sphingolipids for optimal metabolism of vitamins.

1.5.4. Other factors

In addition to dietary fat, vitamin, and toxins, some other components are also known to affect glycosphingolipid metabolism. Dispersion of phosphatidylcholine in the plasma membrane diminishes GM1 incorporation into the cell membrane in human fibroblasts (214). Increasing ionic strength reduces the degradation rate of GD1a in the microsomal membrane (215). Falling pH values accelerates insertion of GD1a into cell membrane (215). Interleukin-1 and TNF-α stimulate SMase activity to release ceramide and phosphorylcholine from sphingomyelin (216). Gangliosides and their derivatives can stimulate or inhibit cell growth. Modulation of sphingolipid metabolism by nutritional or pharmacological strategies has potential to inhibit acute or chronic diseases during development.

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CHAPTER II
RESEARCH PLAN

2.1. RATIONALAE

Gangliosides are ubiquitous and localized at the plasma membrane. These lipids are known to have beneficial effects on cell growth, cell adhesion, immune response, restoration of neuronal cells and receptors for bacterial and viral toxins. Gangliosides may play a critical role in development and protection in the early neonatal period when neonates are completely dependent on supply of nutrients for rapid growth and development. Infants consume gangliosides from mothers' milk, particularly in colostrum, which contains high amounts of gangliosides. It is suggested that gangliosides in milk play important roles in the immune system for protection from food-born and air-born antigens and to enhance neuronal development of cognition and vision. Infant formulas do not contain gangliosides. Chemical and biochemical studies suggest that gangliosides are important lipids for development but it has been not understood how a physiological level of dietary ganglioside plays a role in gut morphology and protection against infection during early development. This research will examine the importance of dietary ganglioside during neonatal development.

2.2. OBJECTIVE

The present research is conducted to determine if dietary ganglioside alters the lipid profile of ganglioside in developing rat intestine, retina and brain and to see the membrane localization in the intestine. The main focus of this study is to determine if dietary ganglioside reduces the inflammatory mediators in developing gut exposed to LPS.

The objectives of this research are to determine:

- if dietary gangliosides can be absorbed by the intestine and alter the lipid profile in intestine, blood, and brain in developing rats.
- the specific localization of ganglioside in the intestinal brush border- or basolateral membrane.
- the effect of dietary gangliosides and LCP on the lipid profile of developing retina.

- how dietary ganglioside alters the phospholipid profile during gut development.
- the bioavailability of dietary ganglioside as a substrate for ether phospholipid synthesis in the intestine.
- the effect of dietary ganglioside on alteration of the lipid profile and inflammatory mediators in intestinal microdomains.
- the influence of dietary ganglioside on structural change in intestinal microdomains.
- anti-inflammatory effects of dietary ganglioside in developing intestine inflamed by LPS.

2.3. HYPOTHESIS

Dietary ganglioside will alter the content and the composition of gangliosides in the intestine and neuronal tissues (retina and brain) through the systematic circulation. Ganglioside will also modify the lipid profile and proteins in the developing intestinal microdomains, thereby having anti-inflammatory effects. It is specifically hypothesized that :

Hypothesis 1: Dietary ganglioside will increase the content of gangliosides and alter the composition of gangliosides in the intestine, blood and brain in developing rats.

Hypothesis 2: Intestinal gangliosides will have a specific localization at the intestinal membrane.

Hypothesis 3: Diet-induced increase of membrane gangliosides will change the ganglioside profile in photoreceptors of the developing retina.

Hypothesis 4: Derivatives of dietary ganglioside will increase phospholipid content and decrease cholesterol content in the intestine.

Hypothesis 5: Derivatives of dietary ganglioside will increase the synthesis of ether phospholipids in the intestine.

Hypothesis 6: Dietary ganglioside will increase ganglioside content and decrease cholesterol content in intestinal microdomains during development.

Hypothesis 7: Diet-induced cholesterol reduction in the intestine will decrease caveolin expression in intestinal microdomains.

Hypothesis 8: Decreased caveolin content in microdomains is associated with decreases in pro-inflammatory mediators, PAF and DG in microdomains.

Hypothesis 9: Dietary ganglioside will inhibit pro-inflammatory signals, PAF, PGE2, LTB4, IL-1β and TNF-α in the intestine and blood inflamed by LPS exposure.

CHAPTER III

DIET-INDUCED CHANGES IN MEMBRANE GANGLIOSIDES IN THE INTESTINAL MUCOSA, PLASMA AND BRAIN

3.1. INTRODUCTION

Gangliosides, sphingolipids with sugar and N-acetyl neuraminic acid (NANA) molecule(s), are found in plasma membranes of mammalian cells and are biologically important molecules involved in cell differentiation, proliferation, neuritogenesis, growth, inhibition, signaling and apoptosis (1-4). De novo biosynthesis of gangliosides is relatively higher during early development than matures (5,6). A simplified diagram of ganglioside biosynthesis from Kolter and Sandhoff (7) is shown (Figure III-1). Ganglioside nomenclature describes the number of N-acetyl neuraminic acid (NANA) groups (M, D, or T refers to mono-, di-, or tri-NANA residues, respectively) as well as the number of sugar moieties attached to the ganglioside (G). Numbers 1, 2, 3, and 4 refer to four, three, two, and one sugars, respectively. Gangliosides can be metabolically interconverted by degradation or biosynthesis of the number of sugars and/or NANA molecules. Ganglioside GM3 is the first ganglioside synthesized from lactosylceramide. GM3 undergoes for GM2 or GD3 synthesis, which characterizes the 'a-' or 'b-' series ganglioside pathway, respectively (7). More complex gangliosides such as GD1a, GT3 and GT1b can be synthesized from GM3 (8).

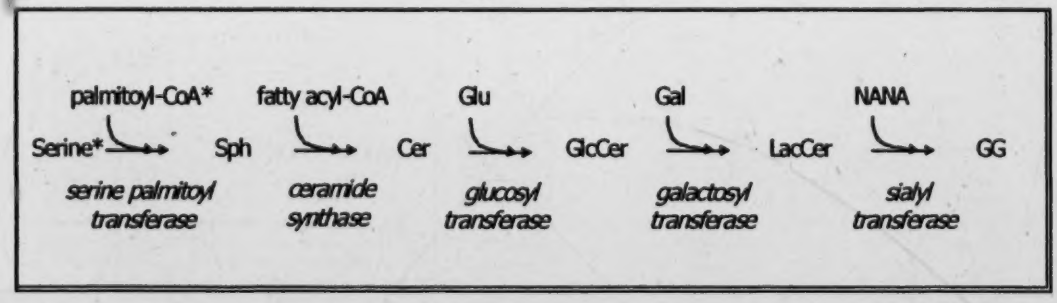

Figure III-1. A simplified diagram of ganglioside biosynthesis adapted from Kolter and Sandhoff (7). * The condensation of serine and palmitoyl-CoA is the rate-limiting step of sphingolipid biosynthesis. Abbreviation: Sph, sphingosine; Cer, ceramide; Glu, glucose; Gal, galactose; GlcCer, glucosylceramide; GalCer, galactosylceramide; NANA, N-acetyl neuraminic acid.

GM3 and GM1 can act as receptors for enterotoxins such as *rotavirus* in animals (9) and *Vibrio cholerae* and *Escherichia coli* in humans (10). GD3 stimulates T-cell activation in human peripheral blood lymphocytes (4,11). Ganglioside content and composition is significantly different between stages of development. For example, in human brain, GM1 increases from birth to the age of 1 year while GD1a decreases in the white matter (5). The ratio of GM3/GD3 is about 0.2~0.3 in human colostrum while the ratio is greater than 3 in mature milk since GM3 gradually becomes a major ganglioside upon lactation (12). Recent studies have also shown neuroprotective effects of GM1. GM1 treatment of neonatal rats prevents hypoxic damage (13). GM1 intervitreally injected is protective against rat retinal ischemia induced by pressure (14), and intravenous administration of GM1 reduces infarct volume caused by focal cerebral ischemia (15). Despite evidence that gangliosides are involved in development, it is still not clear if dietary gangliosides induce changes in membrane gangliosides or where GM3 and GD3 are localized in the enterocyte membrane. This information is vital to understanding the biological functions of these molecules during the period of development in which their role is the most significant.

The cholesterol content of membrane is also important in maintaining an optimal cell membrane environment. Recent work shows that cholesterol homeostasis is related to sphingomyelin content (16), and cholesterol absorption is regulated in part by sphingomyelin content in intestinal cell membranes (17). Cholesterol is enriched in membrane microdomains such as rafts and caveolae, perhaps mediating signal transduction (18). Gangliosides have the same ceramide as the anchored hydrophobic moiety of sphingomyelin, with the only difference occurring in a polar head group. No studies have reported the effect of ganglioside on cholesterol turnover or membrane content of cholesterol in vivo.

The present research was designed to determine if dietary ganglioside increases the content of total and individual gangliosides and affects the level of cholesterol, thereby resulting in change in the ratio of cholesterol to gangliosides in the intestinal mucosa, plasma and brain in developing rats. This is also the first study showing the localization of GM3 and GD3 in the enterocyte membrane.

3.2. MATARIALS AND METHODS

3.2.1. Animals and diets

This study was approved by the University of Alberta Animal Ethics Committee. Male, 18-day-old Sprague Dawley rats (n=8), average body weight 40 ± 4.5 g, were randomly separated into 3 groups of 8, with 2 or 3 rats housed in each polypropylene cage. Animals were maintained at a constant temperature of 23°C and a 12 h light/dark cycle. Animals had free access to water and one of three semi-purified diets containing 20% (w/w) fat for 2 weeks. The composition of the basal diets fed has been reported (Table III-1) (19). Animal body weight and food intake were recorded every other day throughout the experiment. Total food intake was measured for each cage and divided by the number of animals in the cage. The control diet fat was a blend of triglyceride including corn oil, canola oil, coconut oil, and olive oil, which reflected the fat composition of an existing infant formula. Dietary fatty acids were composed of about 31% saturated fatty acids, 48% monosaturated fatty acids and 21% polyunsaturated fatty acids with a ratio of 18:2n-6 to 18:3n-3 of 7.1. Two experimental diets were formulated by adding either sphingomyelin (SM, 1% w/w of total fat, Sigma, MO, USA) or ganglioside (GG, 0.1% w/w of total fat, New Zealand Dairy, New Zealand) to the control diet. Ganglioside-enriched diet consisted of about 0.25% (w/w of total fat) phospholipids and cholesterol was <0.002% w/w of total fat. The ganglioside fraction contained about 80% w/w GD3. GD1b, GM3 and other gangliosides account for 9, 5 and 6% w/w, respectively.

Table III-1. Composition of experimental diets [1]

	Control	SM	GG
Basal diet (g/100g)	80.0	80.0	80.0
Triglyceride	20.0 (100) [3]	20.0 (100)	19.9 (98.9)
Sphingomyelin	-	0.2 (1.0)	-
Ganglioside	-	-	0.02 (0.1)
Phospholipid	-	-	0.05 (0.25)
Cholesterol	-	-	tr [2] (0.002)

[1] The composition of the basal diet has been previously published (19). [2] tr represents trace amount. [3] Values in parenthesis represent the percentage of total fat.

3.2.2. Collection of samples

After anesthetizing animals with halothane, blood was collected by cardiac puncture and immediately spun at 1000 x g (JA 20 Rotor, Beckman, USA) for 30 min to recover plasma. Following decapitation, the brain and small intestine (jejunum to ileum) were excised. The intestine was washed with ice-cold 0.9% saline solution to remove visible mucus and dietary debris, opened and moisture was carefully removed with a paper towel to correctly measure mucosa weight. Intestinal mucosa was scraped off with a glass slide on an ice-cold glass plate. All mucosa samples were weighed and kept in a –70°C freezer until extraction.

3.2.3. Immunofluorescence study

Intestinal sections were collected from animals. Samples were washed with cold phosphate buffered saline (4°C), cut into 5 mm pieces and fixed with 4% paraformaldehyde in PBS for 1 h at 4°C. After washing with cold PBS, samples were infiltrated with 15% and 30% sucrose in PBS for 90 min and overnight, respectively, at 4°C for cryostat protection. The tissue sections were placed on plastic molds and covered with embedding medium by optimal cutting temperature (O.C.T.; Tissue Tek, Sakura Finetek USA) on dry ice. Frozen sections (1 µm thickness) were mounted on polylysine-coated microscope slides and washed in cold PBS for 30 min at room temperature. The sections were blocked with 2% bovine serum albumin in PBS for 1 h at room temperature and then incubated with anti-ganglioside GM3 (Mouse IgM, Seikagaku Co., USA) (diluted 1:25), or anti-ganglioside GD3 monoclonal antibody (Mouse IgM, Seikagaku Co., USA) (diluted 1:25), for 2 h at room temperature. After washing the sections 3 times for 10 min with cold PBS, the sections were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgM (Sigma, MO, USA) (diluted 1:300) for 1 h at room temperature in the dark room and washed with PBS again 3 times for 10 min. After staining, a drop of N-propyl gallate was added onto the section before mounting a cover slip. All samples were sealed with nail polish and examined with a confocal microscope (Zeiss Confocal Laser Microscope 510, Carl Zeiss, Germany) with an Argon laser line (488 nm excitation, barrier filter LP505, Plan-Neofluar 40X, 1.3 oil immersion objective).

3.2.4. Ganglioside extraction and purification

Total lipid was extracted using the Folch method (20). For extracting gangliosides, the lower phase was washed twice with Folch upper phase solution (chloroform/methanol/water, 3:48:47 by vol.). The upper phase gangliosides were pooled and then purified by passing through Sep-Pak C_{18} cartridges (Waters Corporation, Milford, MA, USA) prewashed with 10 mL of methanol, 20 mL of chloroform/methanol (2:1, v/v), and 10 mL of methanol as described by Williams and McCluer (21). The upper phase extract was loaded onto C_{18} cartridges. Cartridges were then washed with 20 mL of distilled water to remove salts and water-soluble contaminants. Gangliosides were eluted with 5 mL of methanol and 20 mL of chloroform/methanol (2:1, v/v), dried under N_2 gas and then redissolved with 500 μL of chloroform/methanol (2:1, v/v). Gangliosides were stored at -70°C until analysis.

3.2.5. Analysis of total and individual ganglioside content by measuring NANA

Total gangliosides were measured as ganglioside-bound NANA (GG-NANA) as described by Suzuki (22). An aliquot of the ganglioside sample purified by Sep-Pak C_{18} cartridges was dried under N_2 gas and dissolved with each of 0.5 mL of distilled H_2O and resorcinol-HCl (23) in screw-capped Teflon-lined tubes. The purple blue color developed by heating was extracted into butylacetate/butanol (85:15, v/v) solvent. Optical density was read by a spectrophotometer (Hewlett Packard, 8452A) at 580 nm. For quantitative analysis, N-acetyl neuraminic acid (Sigma, MO, USA) was used as a standard.

Individual gangliosides were separated by silica gel high performance thin layer chromatography (HPTLC; Whatman Inc, Clifton, NJ, USA) along with standards of ganglioside GM3, GM2, GD3 and bovine brain ganglioside mixture (Alexis, San Diego, CA, USA) in a solvent system of chloroform/methanol/0.2% (w/v) $CaCl_2 \cdot 2H_2O$ (55:45:10, by vol.). Individual ganglioside fractions were scraped off and measured as GG-NANA as described above.

3.2.6. Cholesterol assay

Cholesterol analysis was completed by measuring cholesterol ester and free cholesterol together using a test kit (Sigma, MO, USA). In this methodology, cholesterol

esterase hydrolyzes cholesterol ester to cholesterol and fatty acids. Cholesterol is then oxidized by cholesterol oxidase to cholestenone and hydrogen peroxide. The hydrogen peroxide forms a chromophore which is detectable at 500nm. For the plasma cholesterol assay, plasma was used directly as recommended by the kit. In the tissue cholesterol assay, lipids extracted from tissues were aliquoted into test-tubes and dried under nitrogen. Dried lipids were dissolved with isopropanol and mixed with 100 parts of the assay buffer. Mixed samples were incubated at 37°C for 5 min and color intensity was measured by a spectrophotometer.

3.2.7. Statistical analysis

The values shown are means ± standard deviation (SD). Significant differences between the control group and experimental groups were determined by one-way analysis of variance (ANOVA) with SAS (SAS Institute Inc, Version 8.2, NC, USA). Significant effects of diet treatment were determined by a Duncan multiple range test at a significance level of $P<0.05$. A sample size of six animals was sufficient to achieve a power of 94%. One or two extra animal(s) were added to guarantee the minimum sample size in the event of losses.

3.3. RESULTS

3.3.1. Animal growth and tissues

There were no significant differences among the control, SM and GG groups either in terms of the initial body weight of animals or their final weight after 2 weeks feeding of experimental diets. Brain weight, intestinal mucosal weight and intestinal length were not affected by dietary treatment. Food consumption was not influenced by diet (data not shown).

3.3.2. The localization of GM3 and GD3 in the enterocyte by confocal microscopy

Examination of the localization of GM3 and GD3 in the enterocyte was determined by using a confocal microscope. GM3 stained with FITC-conjugate was almost exclusively localized at the apical membrane of the enterocyte (Figure III-2). The

majority of the GD3 was found in the basolateral membrane of the enterocyte with only minor staining in the apical membrane (Figure III-3).

3.3.3. Total ganglioside content in tissues and plasma

The effect of dietary ganglioside on total ganglioside content of the intestinal mucosa, plasma and brain from animals fed the control and experimental diets for two weeks are shown (Figure III-4). Animals fed the GG diet had significantly higher ganglioside content in the intestinal mucosa, plasma and brain compared to control animals. The highest tissue level of ganglioside was observed in the intestinal mucosal membrane. The lowest level of ganglioside was found in brain membrane. No change in total ganglioside content of either tissues or plasma was found after feeding the SM diet.

3.3.4. Individual ganglioside composition in tissues and plasma

Animals fed the GG diet showed a higher level of GD3 and GQ1b in the intestinal mucosal membrane compared to control animals (Table III-2, $P<0.001$ and $P<0.05$, respectively). This result was accompanied by significant reduction of ganglioside GM3, which is normally a major ganglioside in the intestinal mucosa. Feeding the GG diet did not affect the level of GM2, GM1, GD1a, GD1b or GT1b in the intestinal mucosa compared to the control. Animals fed the SM diet did not exhibit any change in individual ganglioside patterns, but showed increase in GM2 compared to control animals ($P<0.05$).

In plasma, only 4 major ganglioside fractions (GM3, GD1a, GD1b and GT1b) were measured since the total ganglioside content was much lower compared to either tissue. The GD3 fraction could not be quantified because an unknown fraction partially overlapped with GD3. A few minor gangliosides, GM2 and GM1, were faintly visible on the TLC plate. Animals fed the GG or SM diet did not show a significant change in the individual ganglioside composition of plasma compared to control animals. In all cases, GD1a was the major ganglioside in plasma, accounting for 30.2 to 36.0% of the total ganglioside content.

Similar to the ganglioside separation reported by Sonnino et al. (24), brain gangliosides were separated into 14 fractions. No significant change in ganglioside

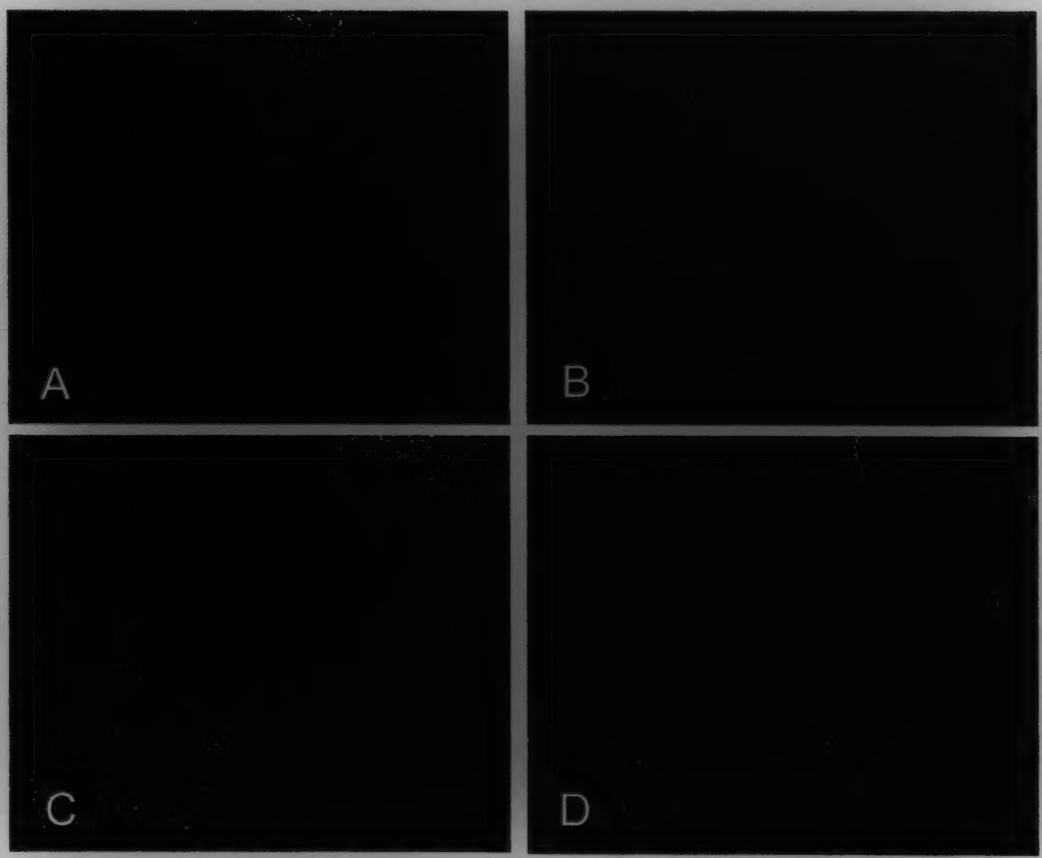

Figure III-2. Immunofluorescent detection of GM3 localization. Immunofluorescent detection of GM3 in intestinal villi was analyzed with (B, D) or without (A, C) treatment of anti-GM3 visualized with FITC-conjugate IgM and confocal microscopy. GM3 was almost exclusively localized at the apical membrane of enterocytes.

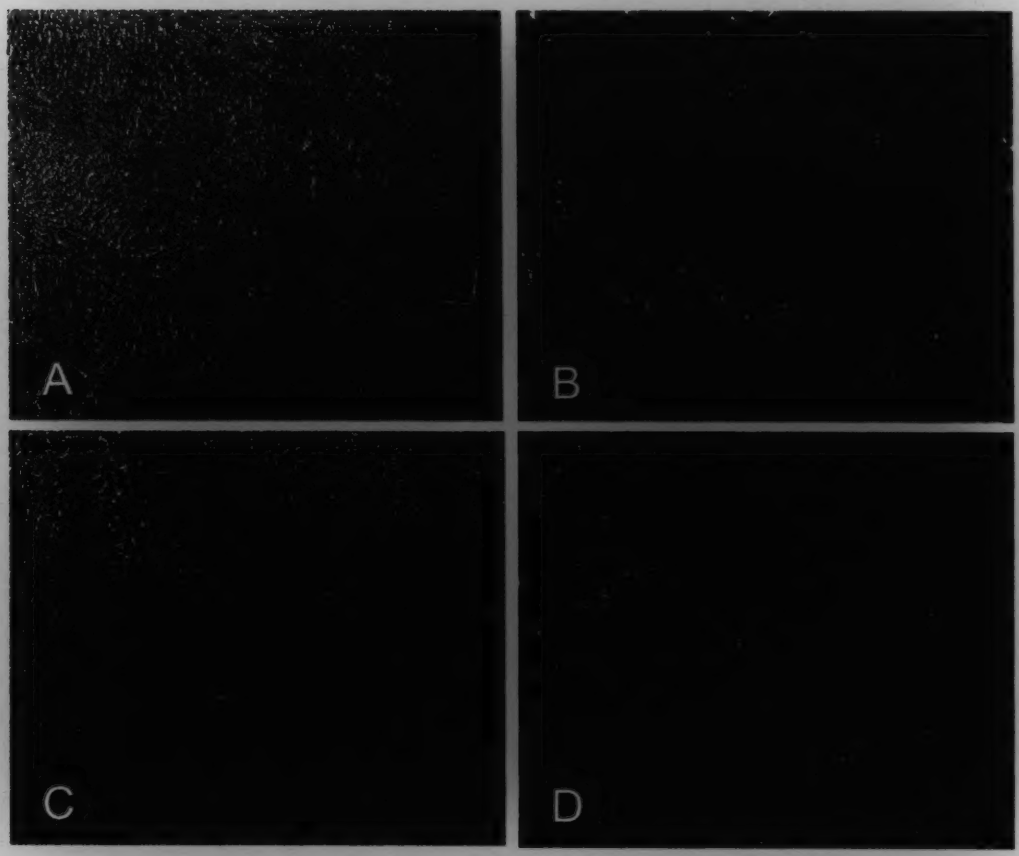

Figure III-3. Immunofluorescent detection of GD3 localization. Immunofluorescent detection of GD3 in intestinal villi was analyzed with (B, D) or without (A, C) treatment of anti-GD3 visualized with FITC-conjugate IgM and confocal microscopy. GD3 was mostly localized at the basolateral membrane with minor staining at the apical membrane of enterocytes.

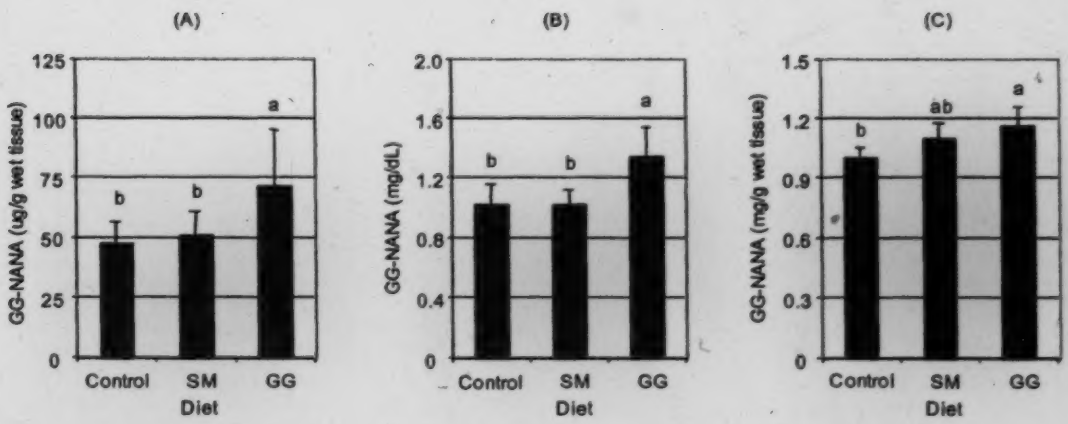

Figure III-4. Effect of dietary treatment on total content of ganglioside-bound NANA (GG-NANA) in the intestinal mucosa (A), plasma (B) and brain (C) for animals fed either the control or experimental diet for two weeks. Values are means ± SD, $P<0.02$ for (A) and (C) and $P<0.003$ for (B), respectively. Treatment values represent the means of n=7, 8 and 6 animals for mucosa, plasma and brain, respectively.

Table III-2. Composition of gangliosides in the rat intestinal mucosa fed either control or experimental diets[1]

Diet	Control	SM	GG
GM3	83.5 ± 6.7^{a}	82.4 ± 7.5^{a}	76.4 ± 6.9^{b}
GM2	2.0 ± 0.9^{b}	4.1 ± 2.4^{a}	2.8 ± 0.8^{ab}
GM1	2.7 ± 1.7	3.0 ± 1.7	1.7 ± 1.2
GD3 [2]	3.2 ± 1.3^{b}	2.2 ± 0.9^{b}	7.5 ± 1.9^{a}
GD1a	2.3 ± 1.5	2.8 ± 1.5	1.9 ± 0.9
GD1b	1.9 ± 1.0	1.5 ± 0.8	2.2 ± 1.4
GT1b	2.1 ± 2.2	1.6 ± 1.6	3.1 ± 2.2
GQ1b	2.3 ± 1.6^{b}	2.6 ± 1.6^{b}	4.5 ± 1.6^{a}

[1] Values are means ± SD of 7 rats and are expressed as a % of total ganglioside fraction. Within a row, values with different superscript letters are significantly different at $P<0.05$. [2] Values are significantly different at $P < 0.001$.

composition was observed between dietary treatment groups. The five major gangliosides in the brain occurred in this order: GD1a>GT1b>GD1b>GQ1b>GM1 and accounted for 70% of the total brain gangliosides in animals fed three different diets. GT1a, GD3 and GM3 contributed to approximately 10% of the total and the other six gangliosides accounted for the remaining 20%. Animals fed the GG diet or SM diet exhibited no change in individual ganglioside composition in the brain compared to control animals, but the total ganglioside content increased.

3.3.5. Cholesterol content in tissues and plasma

Animals fed the GG diet showed a lower level of cholesterol in the intestinal mucosa and brain, but not in the plasma, compared to animals fed the control diet (Figure III-5). Animals fed the SM diet did not exhibit any change in total cholesterol content in the plasma and brain, but exhibited a significant difference in the intestinal mucosa compared to control animals ($P<0.03$). Unlike a previous report (16), the SM diet did not increase cholesterol content in the intestinal membrane, but reduced cholesterol content compared to animals fed the control diet.

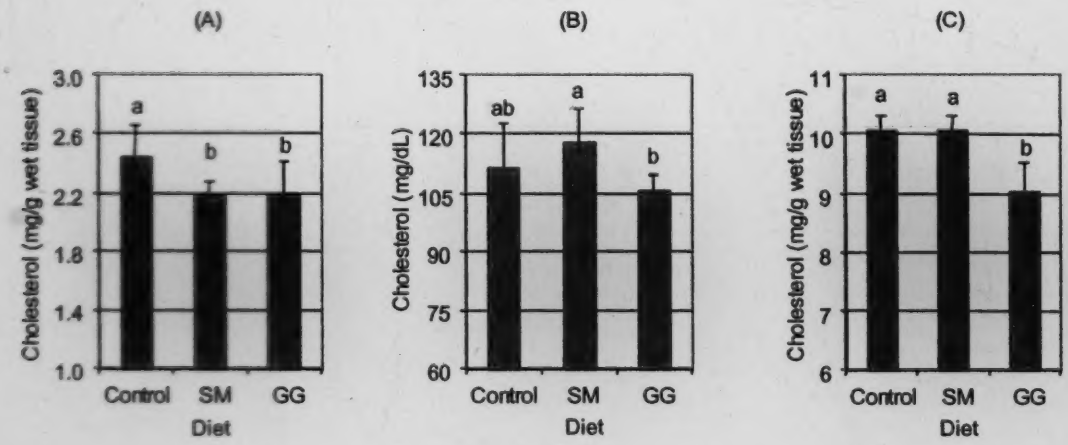

Figure III-5. Effect of dietary treatment on cholesterol content in the intestinal mucosa (A), plasma (B) and brain (C) of animals fed either the control or experimental diets for two weeks. Values are means ± SD, $P<0.03$ for (A) and (B) and $P<0.0002$ for (C). Treatment values represent the means of n=7, 8 and 6 animals for mucosa, plasma and brain, respectively.

3.3.6. Ratio of cholesterol to ganglioside-bound NANA (GG-NANA)

Animals fed the GG diet showed a highly significant reduction in the ratio of cholesterol to GG-NANA in the intestinal mucosa, plasma and brain compared to animals fed the control diet (Figure III-6). Animals fed the SM diet also exhibited a reduced ratio of cholesterol to GG-NANA in the intestinal mucosa and brain, but not in plasma compared to control animals. Of the three dietary treatments, the lowest ratio of cholesterol to GG-NANA was observed in animals fed the GG diet. In contrast, the highest ratio was found in animals fed the control diet in both tissues but not in the plasma. In the plasma, the highest ratio of cholesterol to GG-NANA was observed in animals fed the SM diet.

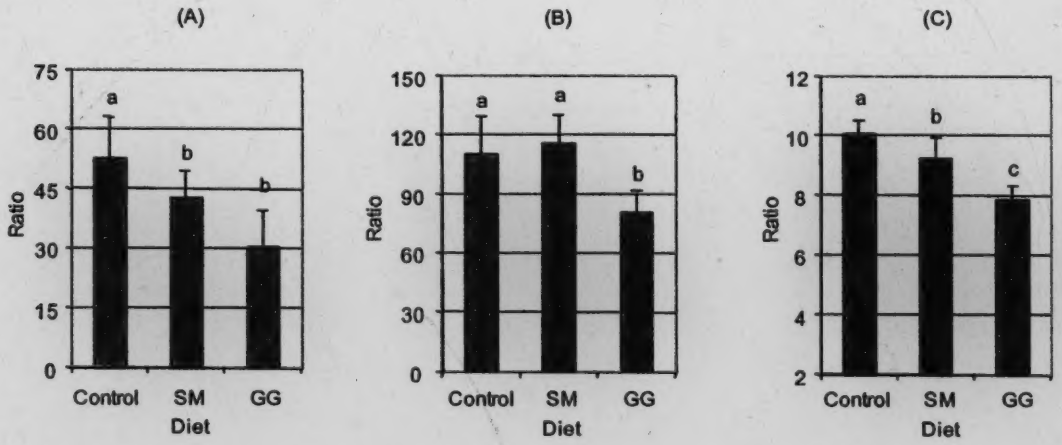

Figure III-6. Effects of dietary treatment on the ratio of cholesterol to ganglioside-bound NANA (GG-NANA) in the intestinal mucosa (A), plasma (B) and brain (C) of animals fed either the control or experimental diets for two weeks. Data values are means ± SD, $P<0.0007$ (A), $P<0.002$ (B) and $P<0.0001$ (C). Treatment values represent the means of n=7, 8 and 6 animals for mucosa, plasma and brain, respectively. The ratio was obtained by dividing the tissue cholesterol content (mg/g wet weight) by the total tissue content of (GG-NANA) (mg/g wet weight).

3.4. DISCUSSION

The notion that gangliosides may have beneficial effects in development has prompted studies of the influence of dietary ganglioside on intestinal and brain

development. GM3 is the major ganglioside in the enterocyte of humans and animals (6, 25) but the intracellular localization is not known. The present study clearly showed that ganglioside GM3 and GD3 are localized at the apical and basolateral membrane of the enterocyte, respectively. This study implies that the different localizations of GM3 and GD3 probably have different biologic and/or physiologic functions for protection and development. The location of GM3 at the apical membrane may support its role in the interception and inactivation of bacterial toxins due to its direct exposure to the lumen. It may function as a first-line defense against infection. GD3 is known to activate the immune system through T-cell function. Situated at the basolateral membrane, GD3 may have access to local immune factors which are present in the lamina propria of the intestine.

GM1 bound with cholera toxin is transcytosized from the apical to the basolateral membrane to activate the basolateral effecter, adenylate cyclase (26). The present study did not examine the possibility that GM3, like GM1, may also be transcytosized. Total ganglioside content and individual ganglioside composition were significantly changed, but the degree of change could not be quantitatively estimated by confocal microscopy. Dietary ganglioside significantly increased membrane ganglioside content in the intestinal mucosa, plasma and brain. Increased membrane ganglioside in the intestinal mucosa might influence enterocyte immune functions since gangliosides have been recently recognized as activators of immune functions (4,11). Neonatal intestinal mucosa has a relatively low level of immunoglobulin-containing cells after birth to about 2 weeks of age (27). Mother's milk and the intestine have a compensatory high level of gangliosides during this period (6,28), suggesting that gangliosides may have a key role in protection of the neonate from antigens (10).

In the present study, increased membrane ganglioside was accompanied by changes in the individual ganglioside composition of the intestinal mucosa. GD3 was increased by feeding gangliosides while the major ganglioside, GM3, decreased compared to control animals. Since GD3 activates T-cells (4,11) and has an anticarcinogenic effect in the mouse colon (29), it is logical to suggest that increased GD3 might influence enterocyte functions and infection by altering the interaction with the developing immune system. In plasma, diet treatment considerably increased total gangliosides, but no change was

found in the composition of individual gangliosides. In contrast to human serum, where GM3 is the major ganglioside (30), GD1a was the major ganglioside in rat plasma. Dietary ganglioside increased total ganglioside content in the brain by 16% compared to animals fed the control diet. The present data suggests that dietary ganglioside may affect brain development since an increase of ganglioside content in the brain may affect protection against neuronal injury (13,15), induce neurite growth (1) and is also observed in well-fed animals compared to undernourished animals (31,32). The present study agrees with previous results describing the pattern of major brain gangliosides (24,33). The lack of significant change in individual ganglioside patterns observed in the brain may be attributed to a short experimental period (two weeks), the lack of change in ganglioside patterns in the plasma or specific control of individual ganglioside composition in the brain. The SM content used in this experiment was relatively much higher (>10 fold) than was the ganglioside content of the GG diet. This higher level of dietary SM did not alter tissue ganglioside content, suggesting that dietary GG is a better source for enhancing membrane GG and that dietary SM may not be used for GG synthesis during the early stage of development.

Cholesterol is an important factor involved in cell permeability, fluidity (34), gap junctions (35) and membrane microdomains called rafts or caveolae (18, 36). Change in membrane cholesterol content in the intestinal mucosa and brain might affect membrane functions during development. The present study indicates that animals fed the GG diet exhibited a significant reduction of cholesterol content in the intestinal mucosa compared to control animals. A disruption in microdomain structures caused by reduced cholesterol content may prevent endocytosis of toxins or invasions by bacteria (37,38). A similar result was also observed for animals fed the SM diet. Long term feeding of 1% sphingolipid in the diet significantly reduces plasma cholesterol content (39). Our study, in agreement with Imaizumi (40), showed that feeding SM for two weeks did not alter plasma cholesterol content. This observation may be due to differences in age, diet, species or in vivo and in vitro experimental conditions.

In the brain, cholesterol is maintained by regulating its de novo synthesis and the uptake of LDL-cholesterol as well as the release of HDL-cholesterol (18,41). Cholesterol synthesis and turnover takes place very slowly in brain relative to liver (42). Dietary

cholesterol and sphingolipids that do not contain NANA have been shown in earlier studies to have no effect on brain cholesterol content (39,43). In our study, significantly lower levels of cholesterol in the brain were observed in animals fed the GG diet compared to animals fed the control diet. This finding suggests that the combination of NANA with dietary glycosphingolipid may be important to the reduction of brain cholesterol.

Reduction in the ratio of cholesterol to GG-NANA in the intestinal mucosa, plasma and brain by feeding the GG diet suggests that dietary ganglioside may alter membrane functions. Early studies show that changes in membrane lipid composition (44) and membrane cholesterol content (34) influence membrane functional properties. The present research also suggests that dietary ganglioside might affect lipid and protein trafficking in membrane microdomains since GM3 and GD3 are localized in different sites and gangliosides and cholesterol are the most abundant lipids present in rafts and caveolae (45, 46). As the functions of microdomains are closely related with ganglioside and cholesterol content (47), changes in the ratio may result in alteration of microdomain structure or function.

In summary, this study suggests that dietary ganglioside is absorbed by the intestine, remodeled in the enterocyte and induces changes in membrane total content of ganglioside and cholesterol in the intestinal mucosa and brain. The observations suggest that dietary ganglioside fed at a physiological level will alter membrane lipid profiles that influence membrane functions involved in a wide variety of cell functions during early development. Infant formulas have much lower levels of gangliosides and a different ganglioside composition compared to that of human breast milk (48,49). The bioavailability of dietary ganglioside demonstrated in this paper and the impact on the lipid composition of developing tissues suggest that these differences in ganglioside levels fed may be of biological importance.

3.5. REFERENCES

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CHAPTER IV

DIETARY GANGLIOSIDE AND LONG-CHAIN POLYUNSATURATED FATTY ACID INCREASE GANGLIOSIDE GD3 CONTENT AND ALTERS THE PHOSPHOLPID PROPILE IN NEONATAL RAT RETINA

4.1. INTRODUTION

An unusual simplified ganglioside composition is observed in adult retinal photoreceptor cells, compared to that in other central nervous system-derived neurons (1). Changes in specific ganglioside content occurs within photoreceptor cells during postnatal maturation to reach an end stage characterized by a predominance of GD3 in the outer retina and only trace amounts of less complex gangliosides (1). The rise in GD3 content corresponds to the period between 10 and 30 days after birth when the outer segments, photoreceptor cells, synaptic cells and rhodposin kinase in the rat retina become functionally active (2,3). Although the majority of gangliosides are localized in the inner retinal membranes, GD3 is primarily found in photoreceptors in the outer retina (1,4), where it plays an important role increasing membrane permeability and fluidity (5,6). Since GD3 is the most prevalent ganglioside in fully mature mammalian retinas (1,7), it can be used as a biological marker to evaluate the stage of retinal development.

Dietary long-chain polyunsaturated fatty acids (LCP) such as docosahexaenoic acid (DHA) and arachidonic acid (AA) influence the lipid composition of retinal membranes, particularly during developmental stages (8-13). Dietary DHA alters the lipid composition of neuronal tissues in retina and brain, affecting the turnover time for rhodopsin in photoreceptor membranes (12,14). Dietary DHA increases DHA levels, and levels of other very long-chain fatty acids in rod outer segment membranes in young rats, whereas it does not change the fatty acid composition of these retinal membranes in mature rats (15,16). Inclusion of DHA and AA in infant formulas improves visual development and acuity in infants (8-10,17), but little is known about the biological basis for this effect. Gangliosides are associated with neuronal cell differentiation and proliferation processes including migration, neurite outgrowth, axon generation, and synapse formation (18-21) processes crucial to visual maturation, but the influence of dietary lipids on retinal ganglioside composition during development is poorly

understood. The present study was conducted to determine whether dietary LCP and ganglioside alters the ganglioside profile in developing rat retina.

4.2 MATERIALS AND METHODS

4.2.1. Animals and diets

This study was approved by the University of Alberta Animal Ethics Committee. Two experimental diets or a control diet were fed to male weanling (18-day-old, 40 $\pm$ 4.5 g) Sprague-Dawley rats for two weeks. Animals had free access to water and one of three semi-purified diets containing 20% (w/w) fat. The control diet was formulated as 80% basal diet plus 20% fat as a triglyceride blend (22), reflecting the overall fat composition of infant formula. The LCP-enriched diet (LCP diet) was formulated by including 1% AA (20:4n-6) and 0.5% DHA (22:6n-3) by weight as triglyceride in the control diet. The ganglioside-enriched diet (GG diet) was formulated by including gangliosides (70~80% GD3, 9% GD1b, 5% GM3, 6% other gangliosides) at a level of 0.1% by weight of total fat. The GG diet also contained 0.25% (w/w of fat) phospholipid and <0.002% (w/w of fat) cholesterol. Body weight and food intake were measured every other day throughout the experimental period.

4.2.2. Collection of retina, lipid extraction and ganglioside separation

After decapitation of animals, whole retinas were removed. All samples were weighed and kept in a -70°C freezer until analysis. Total lipids were extracted using the Folch method (23). Gangliosides were extracted into the Folch upper phase solution. The lower organic phase was washed twice with chloroform/methanol/water (3:48:47, by vol) and the upper phase extracts combined. Gangliosides were purified by passing the upper phase extract through Sep-Pak C_{18} cartridges (Waters Corporation, Milford, MA) preconditioned with 10 mL of methanol, 20 mL of chloroform/methanol (2:1, v/v), and 10 mL of methanol. Cartridges were then washed with 20 mL of distilled water to remove salts and water-soluble contaminants. Gangliosides were eluted with 5 mL of methanol and 20 mL of chloroform/methanol (2:1, v/v), dried under N_2 gas and then redissolved with 500 µL of chloroform/methanol (2:1, v/v). Gangliosides were stored at -70°C until analysis.

4.2.3. Analysis of ganglioside content

Measurement of total gangliosides as ganglioside bound-N-acetyl neuraminic acid (GG-NANA) was performed as described by Suzuki (24). An aliquot of the purified ganglioside sample was dried under N_2 gas and dissolved with 0.5 mL each of distilled H_2O and resorcinol-HCl (25). The purple-blue color developed by heating was extracted into butyl acetate/butanol (85:15, v/v). Optical density was read at 580 nm. Commercial NANA (Sigma, MO) was used as a standard.

Individual gangliosides were separated by silica gel high performance thin layer chromatography (HPTLC; Whatman Inc, Clifton, NJ) in a solvent system of chloroform/methanol/0.2 % (w/v) $CaCl_2 \cdot 2H_2O$ (55:45:10, by vol) and identified using ganglioside standards GM3, GM2, GD3 and bovine brain ganglioside mixture (Alexis, San Diego, CA). Individual gangliosides were recovered and measured as described above.

4.2.4. Analysis of phospholipid content

Phospholipids were separated from total retinal lipids by thin layer chromatography (TLC) on silica gel 'G' (Fisher Scientific, CA) using chloroform/methanol/water, (65:35:6, by vol.). Individual phospholipids were resolved on silica gel 'H' TLC plates using chloroform/methanol/2-propanol/0.2% KOH/triethylamine (45:13.5:37.5:9:27, by vol). and identified by comparison to authentic phospholipid standards (Sigma, MO). Plates were visualized by 0.1 % anilinonaphthalene sulfonic acid under UV exposure. Lipid fractions were recovered and lipid phosphate was measured according to the method of Itoh *et al.* (26)

4.2.5. Statistical analysis

Six retinas from three animals were pooled to constitute one replicate to analyze retinal lipids because of the minute amount of lipids in the retina. Values presented are means ± standard deviation (SD) from 6 replicates (n=6) except for individual phospholipid analysis (n=5). Significant differences between the control group and experimental groups were determined by one-way analysis of variance (ANOVA) with

SAS (SAS Institute Inc, Version 8.2, NC, USA). Significant effects of diet treatment were determined by a Duncan multiple range test at a significant level of $P<0.05$.

4.3. RESULTS

4.3.1. Animal growth and diet consumption

The initial and final body weight of animals or food consumption after two weeks feeding of experimental diets was not significantly different among control, LCP and GG diet group. Retina weight was not affected by dietary treatment (data not shown).

4.3.2. Ganglioside content and composition

Animals fed the GG diet showed higher levels of total gangliosides in the retina by 39% ($P<0.0008$) when compared to animals fed the control diet (Figure IV-1-A). Feeding animals either the LCP or the GG diet caused an increase in the relative percentage of GD3 in the retina in comparison with feeding animals the control diet (by 19% and 13%, respectively; Table IV-1). The composition of GM3, GM1, GD1a, GD1b and GT1b was not changed by either experimental diet (Table IV-1).

4.3.3. Phospholipid content and composition

Feeding animals the LCP diet, but not the GG diet, reduced total phospholipid content in the retina compared to animals fed the control diet (Figure IV-1-B). Animals fed either the LCP diet or the GG diet showed lower levels of phosphatidylinositol and lyso-phosphatidylethanolamine (PE) and higher levels of phosphatidylserine and phosphatidylcholine (PC) compared to animals fed the control diet (Table IV-2). PE and sphingomyelin (SM) were not changed by either diet treatment. Ratios of PE to PC, major phospholipids in the retina, and PC to SM were not affected by either the LCP or the GG diet treatment (Table IV-2).

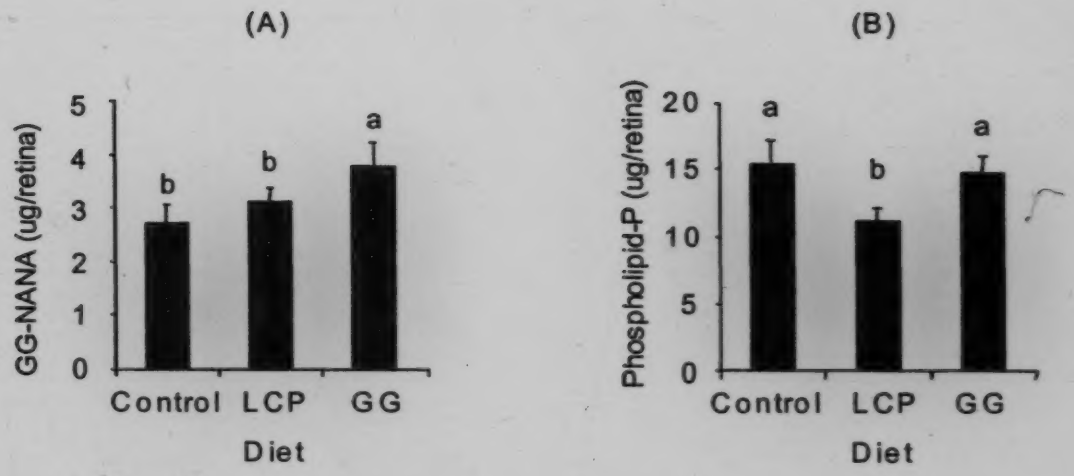

Figure IV-1. Total content of (A) gangliosides and (B) phospholipids in the retina of control and treatment groups. Values are the means ± SD of 6 samples. Letters represent a significant difference between groups at levels of $P<0.0008$ and $P<0.001$, respectively.

Table IV-1. The composition of gangliosides in the retina of animals fed different diets[1]

Ganglioside (%)	Control	LCP	GG	Diet effect (P)
GM3[2]	7.6 ± 2.8	5.8 ± 3.2	7.7 ± 2.3	
GM1	7.3 ± 1.8	6.8 ± 2.0	7.7 ± 2.2	
GD3	25.0 ± 1.8[b]	29.8 ± 1.7[a]	28.2 ± 3.5[a]	0.01
GD1a	14.3 ± 3.3	15.4 ± 3.5	16.1 ± 1.8	
GD1b	19.1 ± 2.2	19.0 ± 42.	16.7 ± 1.0	
GT1b[3]	26.9 ± 2.9	23.2 ± 3.1	23.6 ± 1.3	

[1] Values are means ± SD where n=6 for each group. Percentage of gangliosides was presented as % of total NANA content (µg/retina) in the ganglioside fraction.
[2] Nomenclature of gangliosides is described by Svennerholm (27).
[3] GT1b fraction included GQ1b fraction because of close proximity during TLC separation.

Table IV-2. The composition of phospholipids in the retina of animals fed different diets[1]

Phospholipid (%)	Control	LCP	GG	Diet effect (P)
PE[2]	35.2 ± 4.7	33.4 ± 1.5	34.4 ± 2.3	
PI	7.4 ± 1.6[a]	5.1 ± 1.3[b]	4.6 ± 0.7[b]	0.01
PS	2.3 ± 0.4[b]	2.8 ± 0.4[a]	3.1 ± 0.1[a]	0.01
LPE	8.3 ± 0.6[a]	6.6 ± 0.4[b]	6.4 ± 1.0[b]	0.001
PC	40.4 ± 3.3[b]	45.5 ± 2.8[a]	45.6 ± 2.7[a]	0.04
SM	6.5 ± 1.3	6.6 ± 2.2	6.0 ± 0.7	

[1] Values are means ± SD where n=5 for each group. [2] Percentage of phospholipids was presented as phosphate content (μg/retina) in the phospholipid fraction.

4.4. DISCUSSION

The present study demonstrates for the first time that dietary gangliosides and LCP can modify the ganglioside composition in the developing retina. Although dietary LCP has long been known to modify the lipid classes and the composition of phospholipids in the developing retina (28,29) as well as to improve visual function (30,31), an explanation for these effects at a metabolic level was lacking. This study found that animals fed the LCP diet showed higher relative levels of GD3, but total ganglioside content was not changed, compared to animals fed the control diet. The effect of dietary LCP on the proportional increase of GD3 may suggest that dietary LCP influence activity of GD3 synthase, an enzyme in the outer retina required to synthesize GD3 from GM3 (32). Trafficking of DHA-containing PL from the trans-Golgi network to the retina outer segment is accompanied with rhodopsin (33). Sphingolipids including GG are enriched in microdomains called lipid rafts or caveolae, which are important domains for lipid trafficking (34-37). Thus, the present study suggests that increased levels of GD3 in response to the LCP diet may influence the intracellular trafficking of DHA and rhodopsin during retinal development. This potential interaction suggests one possible mechanism to explain the improvement in visual acuity associated with dietary LCP.

In the retina of the rat, the outer segments, photoreceptor cells, synaptic cells and rhodopsin kinase become functionally active between 10 days and 1 month after birth (2,38) during which time GD3 becomes the predominant ganglioside (1,4,7). GD3 in the outer retina is involved in increasing membrane permeability and fluidity (5,6) and is enriched in differentiated retinas (1,7). Since animals used in the present study were fed for two weeks starting from 17 days of age, this study suggests that dietary LCP and GG may stimulate retinal maturation and development by increasing GD3 content.

Concurrent with changes in ganglioside profile was an alteration in retinal phospholipid composition attributed to both the GG and the LCP diets. Both diets were associated with decreases in the relative amounts of phosphatidylinositol and lyso-phosphatidylethanolamine, and increases in phosphatidylserine and phosphatidylcholine (Table IV-2). There was no effect of the GG diet on total phospholipids, whereas the LCP diet was associated with a decrease in total retinal phospholipids (Figure IV-1-B). Phospholipid turnover alters electric surface potential by affecting calcium and cation concentration in retinal rod outer segments (39) and is tightly regulated by light and phosphorylation-dephosphorylation reactions (40). Thus, compositional changes in retinal phospholipids in response to dietary ganglioside or LCP may affect light adaptation and activation of protein kinases, which ultimately may lead to enhanced development of retinal function in neonates.

In summary, this study demonstrates that dietary LCP and gangliosides modify metabolism of gangliosides and phospholipids in developing retinal membranes. The present experiment suggests that a small physiologic amount of phospholipids or gangliosides have profound effects on the lipid profile of membranes in the retina. The bioavailability of gangliosides in the diet is high such that GD3 composition in structural components of the photoreceptor membrane is rapidly altered. Further investigation is needed to determine whether dietary gangliosides alter ganglioside content in other neuronal cell types.

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CHAPTER V

ALTERATION OF THE PHOSPHOLIPOID PROFILE BY DIETARY GANGLIOSIDE IN RAT INTESTINAL MUCOSA DURING DEVELOPMENT

5.1. INTRODUCTION

The intestinal membrane undergoes dramatic changes in the lipid profile during postnatal development throughout cell migration from crypt to villous membranes (1) and from birth to adulthood (2). Total phospholipid content is much higher in newborn microvillus membranes compared to that of adults (3). Phosphatidylcholine (PC) increases while phosphatidylinositol (PI) decreases in newborn rats compared to adult rats (4). 18:2n-6 and 18:3n-3 are enriched in villous membranes by 2-fold compared to crypt membranes (5). During the postnatal development, many digestive enzymes and transporters become active (6), and are influenced by its lipid composition and dietary fats (7-9).

Phospholipids play multiple roles within membranes by forming stable bilayers and being used for an enzyme activator such as Ca^{2+}-ATPase (10), Na^{+}/K^{+}-ATPase (11) and glucose transporter (12,13). In order to activate Ca^{2+}-ATPase enzyme, phospholipids seem to be of primary importance because 30 phospholipid molecules are required per molecule of ATPase (14). Intestinal fluidity, permeability, enzymes, transport systems, and cation binding and release are altered by changing the fatty acid composition and content of phospholipids surrounding proteins (15-18).

Glycosphingolipids including gangliosides are also known to have developmental changes during gut development (19,20). Twenty to thirty percent of the total lipids of rat intestine are glycosphingolipids, one third of which are gangliosides (21). The total content of glycosphingolipids in microvillus membranes is four times higher compared to whole mucosa (21). GM3 is the major ganglioside in the rat intestine and is mostly localized at differentiated microvillus cells compared to undifferentiated crypt cells (22). GM3 localizes at the brush border membrane while GD3 is found at the basolateral membrane (23). Intestinal GM3 reaches maximum level at 6 days of birth and quickly falls over 2 weeks (19), suggesting an importance of gangliosides in the early stage of development. Gangliosides are known to play roles in cell adhesion and signaling

transduction by modulating growth factor receptors (24, 25). Ganglioside GM1 acts as *Cholera* and *E.coli* toxin receptors in the intestine (26). A recent animal study demonstrates that dietary ganglioside influences the maturation of the intestinal immune system (27). Animals fed dietary ganglioside increase the number of Th1 and Th2 cytokine-secreting lymphocytes in lamina propria and Peyer's patches lymphocytes in mice (27). Gangliosides also contribute in several ways to modulate complex ion channels, transport/exchange proteins, Ca^{2+}-utilizing enzymes and neurotransmitters (28, 29). Thus, it is suggested that high amount of gangliosides in milk and intestine in the early stage of development act as an inhibitor of enterotoxin (30,31), an activator for the immune system (27) and a modulator of channels/transporters (28,29).

There are a number of ways dietary lipids modify intestinal lipid profile, morphology and nutrient transport in developing rats (32-35). The relationship between gangliosides and phospholipids remains largely unexplored. Previous studies show that derivatives of sphingolipids are hexadecanal, a precursor of fatty alcohols and fatty acids, ethanolamine and choline (36,37). This suggests a possible metabolic connection between sphingolipids and phospholipids. Developing animals and infants consume dietary ganglioside from mothers milk (38) but it is not clear how dietary ganglioside affects the phospholipid profile during development. The present study showed the metabolic conversion of dietary ganglioside during gut development to phospholipids and alteration of the fatty acid composition of total and individual phospholipids.

5.2. MATERIALS AND METHODS

5.2.1. Animals and diets

This study was approved by the University of Alberta Animal Ethics Committee. Male 18-day-old Sprague Dawley rats (n=6), average body weight 40 ± 4.5 g, were randomly separated into 3 groups of 8 with 2 or 3 rats housed in each polypropylene cage. Animals were maintained at a constant temperature of 23°C and a 12 h light/dark cycle. Animals had free access to water and one of three semi-purified diets containing 20% (w/w) fat for 2 weeks. The composition of the basal diets fed has been reported (39). Animal body weight and food intake were recorded every other day throughout the experiment. The control diet fat was a blend of triglyceride, which was similar to the fat

composition of an existing infant formula. Dietary fatty acids were composed of about 31% saturated fatty acids, 48% monounsaturated fatty acids and 21% polyunsaturated fatty acids with a ratio of 18:2n-6 to 18:3n-3 of 7:1. Two experimental diets were formulated by adding either sphingomyelin (SM, 1.0% w/w of total fat, Sigma, MO, USA) or ganglioside (GG, 0.1% w/w of total fat, New Zealand Dairy, New Zealand) to the control diet. In the GG diet, the ganglioside fraction contained about 80% w/w GD3, with GD1b, GM3 and other gangliosides accounting for 9, 5 and 6% w/w, respectively. Cholesterol was <0.002% w/w of total fat. In order to compare a bioavailability of gangliosides, sphingomyelin, a sphingolipid without sugars and N-acetyl neuraminic acids but a phosphorylcholine, was chosen as a secondary control.

5.2.2. Collection of samples

After anesthetizing animals with halothane, small intestine (jejunum to ileum) was excised. The intestine was washed with ice-cold 0.9% saline solution to remove visible mucus and dietary debris, opened longitudinally and moisture was carefully removed to correctly measure mucosa weight. Intestinal mucosa was scraped off with a glass slide on an ice-cold glass plate. All mucosa samples were weighed and kept in a –70°C freezer until extraction.

5.2.3. Lipid extraction and phospholipid content analysis

Total lipid was extracted by using the Folch method (40). For extracting total lipid, the intestinal mucosa was washed twice with theoretical Folch lower phase solution (chloroform/methanol/ water, 86:14:1, by vol). The lower phase lipid was pooled, dried and then dissolved in chloroform:methanol (2:1, v/v). Extracted lipid from the intestine was applied onto a silica gel 'G' TLC plate (Fisher Scientific, CA) using chloroform/methanol/water, (65:35:6, by vol) and a silica gel 'H' TLC plate using chloroform/methanol/2-propannol/0.2% KOH/triethylamine, 45:13.5:37.5:9:27, by vol) for total and individual phospholipids, respectively. A commercial phospholipid standard (Sigma, MO, USA) was also spotted on the plate for individual phospholipid identification. After development, TLC plates were dried, sprayed with 0.1 % ANSA (anilino naphthalene sulfonic acid) and exposed under UV light to detect total and

individual phospholipids. Lipid fractions identified were then recovered and lipid phosphate was measured by a spectrophotometer (Hewlett Packard, 8452A) at 790 nm (41).

5.2.4. Analysis of fatty acid composition of phospholipids

The fraction of individual phospholipids was identified by TLC separation. Fatty acid methylesters were prepared with 3N-methanolic-HCl (Supelco, PA, USA) for 16 hr at 70°C. The transesterfied fatty acid composition of the fractions was analyzed by GLC equipped with a flame ionization detector and BP-20 fused capillary column (SGE, Australia). The flow rate of helium gas was 1.6 mL/min and the oven, injector and detector temperatures were 200°C, 250°C, and 250°C, respectively.

5.2.5. Statistical analysis

The values presented are means ± standard deviation (SD) from 6 animals. Significant differences between the control group and experimental groups were determined by one-way analysis of variance (ANOVA) with SAS (SAS Institute Inc, Version 8, NC, USA). Significant effects of tissues or diets in a grand mean were determined by a Duncan multiple range test at a significance level of $P < 0.05$.

5.3. RESULTS

5.3.1. Content of total phospholipid

Animals fed the GG diet showed a significant decrease in total phospholipid content in mucosa by 23% compared to animals fed the control diet (Figure V-1). Feeding the SM diet animals did not change the total content of phospholipids compared to either the control diet or GG diet.

5.3.2. Composition of individual phospholipids

In a comparison of individual phospholipids (Table V-1), animals fed dietary ganglioside significantly decreased phosphatidylcholine (PC) in mucosa. Feeding animals the SM diet showed more significant changes in phospholipids such as an increase in SM

(38%) and phosphatidylethanolamine (PE, 20%) and a decrease in PC (19%) compared to animals fed the control diet.

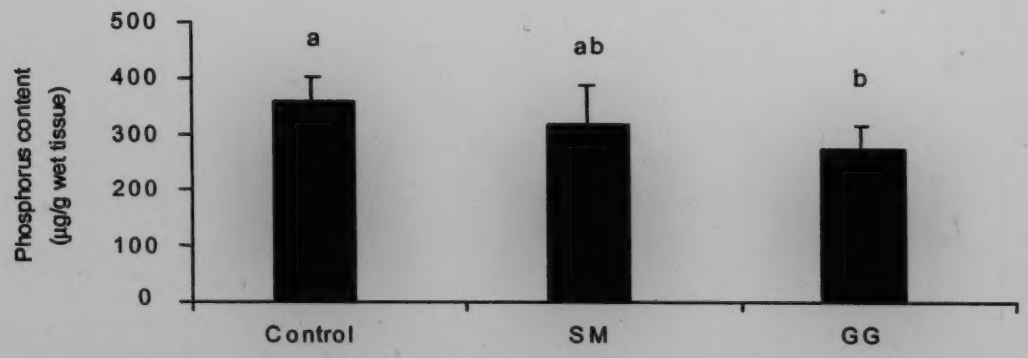

Figure V-1. The content of total phospholipids in intestinal mucosa from animals fed the control, SM or GG diet for 2 weeks. Total phospholipid was separated on silica gel 'G' TLC plate using a solvent system, petroleum ether/diethylether/acetic acid, (80:20:1, by vol). After 0.1% ANSA (anilino naphthalene sulfonic acid) spray, the phospholipid fraction was identified and recovered for measuring phosphate content in phospholipids as described (42). Optical density was read in a spectrophotometer at 790 nm. Statistical analysis was an ANOVA with a Duncan multiple range test at $P<0.05$.

Table V-1. The composition of phospholipids in intestinal mucosa from animals fed the control or experimental diets for 2 weeks [1]

Lipids	Control	SM	GG	Diet effect (P)
LPC	4.9 ± 0.6	6.6 ± 2.2	5.6 ± 0.6	
SM	7.9 ± 1.0^{b}	10.8 ± 2.5^{a}	8.6 ± 2.2^{ab}	0.05
PC	48.5 ± 2.2^{a}	39.1 ± 2.8^{c}	45.0 ± 3.0^{b}	0.0001
PS	9.0 ± 0.6	8.9 ± 2.2	9.4 ± 2.0	
PI	4.9 ± 0.7	4.8 ± 1.8	4.6 ± 1.1	
PE	24.9 ± 2.4^{b}	29.8 ± 1.5^{a}	26.9 ± 2.5^{b}	0.01

[1]Values are means ± SD of 6 animals from each group. Percentage of phospholipid was calculated by phosphorous in individual phospholipid.

5.3.3. Fatty acid composition of individual phospholipids

5.3.3.1. Lyso-phosphatidylcholine (LPC)

Animals fed dietary ganglioside increased 14:0 and 20:5n-3 with decreases in MUFA, 22:1, 22:2 and 24:1 compared to animals fed the control diet (Table V-2). SM diet exhibited increases in 20:5n-3 and 18:2 and decreases in 20:0, 22:1, 22:2, and 24:1. Animals fed both sphingolipid diets showed a significant increase in 20:5n-3 (about 7~10 fold, $P<0.0001$) but decreases in 22:2 and 24:1 compared to control animals.

5.3.3.2. Sphingomyelin (SM)

Animals fed dietary ganglioside had significant changes in SFA such as increases in 22:0, 23:0 and 24:0 and a decrease in 16:0 compared to animals fed the control diet (Table V-3). Decreased 18:2n-6 and 20:3n-6 was also observed. Feeding animals the SM diet did not increase SFA, 22:0, 23:0 and 24:0, but animals exhibited decreases in 16:0, 18:2 and 22:4n-6 compared to control animals. Both dietary sphingolipids decreased ∑PUFA compared to the control diet. This change resulted in a significant increase in the ∑SFA/∑PUFA ratio of the SM fraction, (28 *vs* 51 and 28 *vs* 55, for the SM and GG diet, respectively), compared to control animals ($P<0.01$).

5.3.3.3. Phosphatidylcholine (PC)

Feeding animals dietary ganglioside significantly increased SFA, 18:0, 20:0, and 24:0 and PUFA, 20:4n-6, and 22:6n-3 in PC compared to the control diet (Table V-4). Animals fed dietary ganglioside decreased 18:2n-6, 20:2, and 24:1 but increased the level of SFA. Animals fed the SM diet did not increase SFA but decreased 24:1 compared to animals fed the control diet.

5.3.3.4. Phosphatidylserine (PS)

When animals were fed with dietary ganglioside, SFA (14:0 and 16:0) and PUFA (20:4n-6 and 20:5n-3) were significantly increased and 20:3n-6, 22:2 and 24:1 were significantly decreased compared to animals fed the control diet (Table V-5). Animals

Table V-2. Fatty acid composition of LPC in intestinal mucosa fed the control or sphingolipid diets in rats[1]

Fatty acid	Control	SM	GG	Diet effect (P)
C 14:0	2.8±1.1 [ab]	1.7± 0.7 [b]	3.8± 1.2 [a]	0.05
C 16:0	31.0±4.4	30.4± 3.3	28.7± 2.7	
C 18:0	46.2±5.6	48.8± 4.3	48.3± 3.3	
C 18:1(9)	6.4±0.5	6.4± 1.4	6.1± 2.7	
C 18:2(6)	3.2±0.7 [b]	4.5± 1.0 [a]	3.3± 0.5 [b]	0.05
C 20:0	0.7±0.4	0.9± 0.1	1.0± 0.2	
C 20:1(9)	0.3±0.2	0.2± 0.1	0.2± 0.2	
C 20:4(6)	1.0±0.3	1.1± 0.6	1.3± 0.1	
C 20:5(3)	0.2±0.1 [c]	1.5± 0.3 [b]	2.0± 0.3 [a]	0.0001
C 22:0	1.1±0.1 [ab]	0.9± 0.1 [b]	1.2± 0.3 [a]	0.05
C 22:1(9)	0.5±0.1 [a]	0.2± 0.1 [b]	0.3± 0.1 [b]	0.001
C 22:2	0.6±0.3 [a]	0.1± 0.1 [b]	tr [3] ± 0.0 [b]	0.001
C 22:4(6)	0.8±0.4	0.4± 0.1	0.7± 0.4	
C 24:0	1.1±0.8	1.4± 0.4	1.8± 0.4	
C 22:6(3)	0.8±0.3	0.6± 0.2	0.7± 0.3	
C 24:1(9)	3.3±2.0 [a]	0.9± 0.3 [b]	0.8± 0.2 [b]	0.01
ΣSFA[2]	82.9±2.7	84.2± 1.7	84.7± 2.7	
ΣMUFA	10.4±2.1	7.7± 1.6	7.3± 2.9	
ΣPUFA	6.6±1.6	8.1± 1.1	8.0± 0.6	
ΣSFA/ΣPUFA	13.2±3.6	10.6± 1.5	10.7± 0.7	

[1] Values are means ± SD of 6 rats. [2] SFA, MUFA and PUFA stand for saturated fatty acids, mono-unsaturated fatty acids and poly-unsaturated fatty acids, respectively. [3] tr means values less than 0.05%.

Table V-3. Fatty acid composition of SM in intestinal mucosa fed the control or sphingolipid diets in rats[1]

Fatty acid	Control	SM	GG	Diet effect (P)
C 14:0	1.9± 0.7	1.7 ± 0.4	2.4±0.9	
C 16:0	61.0± 2.1[a]	55.4 ± 6.1[b]	54.4±3.9[b]	0.05
C 18:0	9.1± 0.4	10.8 ± 3.1	10.0±0.9	
C 18:1(9)	2.9± 1.1	4.7 ± 4.9	2.3±0.7	
C 18:2(6)	1.0± 0.2[a]	0.6 ± 0.1[b]	0.7±0.1[b]	0.001
C 20:0	3.2± 0.6[b]	4.4 ± 1.1[a]	4.1±0.5[ab]	0.05
C 20:3(6)	0.8± 0.4[a]	0.4 ± 0.2[ab]	0.1±0.2[b]	0.01
C 20:4(6)	0.4± 0.1	0.4 ± 0.1	0.3±0.1	
C 20:5(3)	0.0± 0.0	0.0 ± 0.0	0.0±0.0	
C 22:0	5.0± 0.6[b]	5.2 ± 0.8[b]	6.9±0.9[a]	0.01
C 22:1(9)	0.2± 0.2	0.5 ± 0.4	0.2±0.1	
C 22:2	0.4± 0.3	0.2 ± 0.1	0.2±0.1	
C 22:4(6)	0.6± 0.2[a]	0.2 ± 0.2[b]	0.5±0.2[a]	0.001
C 23:0	2.3± 0.2[b]	1.8 ± 0.5[b]	3.1±0.6[a]	0.01
C 24:0	6.0± 0.5[b]	7.3 ± 1.4[b]	9.1±1.1[a]	0.001
C 22:6(3)	0.0± 0.0	0.0 ± 0.0	0.0±0.0	
C 24:1(9)	5.3± 0.8	6.3 ± 1.0	6.0±0.7	
ΣSFA[2]	88.4± 1.9	86.7 ± 4.7	89.8±1.7	
ΣMUFA	8.4± 1.8	11.5 ± 4.5	8.5±1.4	
ΣPUFA	3.3± 0.6[a]	1.8 ± 0.4[b]	1.8±0.5[b]	0.001
ΣSFA/ΣPUFA	28.1± 5.7[b]	50.6 ± 12.4[a]	54.6±14.6[a]	0.01

[1] Values are means ± SD of 6 rats. [2] SFA, MUFA and PUFA stand for saturated fatty acids, mono-unsaturated fatty acids and poly-unsaturated fatty acids, respectively.

Table V-4. Fatty acid composition of PC in intestinal mucosa fed the control or sphingolipid diets in rats[1]

Fatty acid	Control	SM	GG	Diet effect (P)
C 14:0	0.9± 0.3	0.6± 0.4	0.7± 0.2	
C 16:0	22.8± 0.8	20.3± 3.0	20.7± 1.7	
C 18:0	22.3± 0.6^{b}	23.5± 1.4^{ab}	24.3± 1.4^{a}	0.05
C 18:1(9)	10.7± 0.8	10.8± 0.9	9.8± 0.7	
C 18:2(6)	28.5± 0.7^{a}	29.1± 1.1^{a}	26.2± 2.1^{b}	0.01
C 18:3(3)	0.2± 0.1	0.2± 0.1	0.2± 0.0	
C 20:0	0.6± 0.2^{b}	0.7± 0.2^{ab}	1.0± 0.2^{a}	0.05
C 20:1(9)	0.5± 0.1	0.5± 0.1	0.4± 0.1	
C 20:2	0.2± 0.1^{a}	0.3± 0.0^{a}	0.1± 0.1^{b}	0.01
C 20:3(6)	0.4± 0.0	0.4± 0.1	0.4± 0.1	
C 20:4(6)	9.4± 0.9^{b}	10.5± 1.7^{b}	12.2± 0.9^{a}	0.01
C 20:5(3)	0.3± 0.1	0.4± 0.2	0.4± 0.2	
C 22:0	0.5± 0.2	0.6± 0.2	0.8± 0.2	
C 22:1(9)	0.2± 0.0	0.2± 0.0	0.1± 0.1	
C 22:2	0.1± 0.1	0.1± 0.1	0.1± 0.0	
C 22:4(6)	0.2± 0.1	0.1± 0.0	0.1± 0.1	
C 24:0	0.4± 0.1^{b}	0.4± 0.1^{b}	0.6± 0.2^{a}	0.01
C 22:6(3)	1.5± 0.2^{b}	1.5± 0.3^{b}	1.8± 0.2^{a}	0.05
C 24:1(9)	0.5± 0.2^{a}	0.3± 0.2^{b}	0.3± 0.1^{b}	0.01
∑SFA[2]	47.8± 1.2	46.1± 2.1	47.6± 2.8	
∑MUFA	11.9± 0.9^{a}	11.6± 0.9^{ab}	10.6± 0.6^{b}	0.05
∑PUFA	40.6± 1.1	42.4± 2.0	41.4± 2.3	
∑SFA/∑PUFA	1.2± 0.1	1.1± 0.1	1.2± 0.1	

[1] Values are means ± SD of 6 rats. [2] SFA, MUFA and PUFA stand for saturated fatty acids, mono-unsaturated fatty acids and poly-unsaturated fatty acids, respectively.

showed a reduced level of 20:3n-6, 22:2 and 24:1. In animals fed the SM diet, the level of 20:5n-3 and 24:0 was increased with concomitant decreases in 20:2 and 22:2 compared to control animals. Overall, both sphingolipid diets increased 20:5n-3 by 5~6 fold ($P<0.0001$) but decreased 22:2 compared to the control diet.

5.3.3.5. Phosphatidylinositol (PI)

Animals fed the GG diet showed a high level of 14:0, 20:5n-3 and 24:0 and a low level of 18:1, 20:2, and 24:1 compared to animals fed the control diet (Table V-6). Feeding animals the SM diet increased 20:5n-3 and 24:0 but decreased MUFA (18:1, 22:1 and 24:1) and 20:2 compared to control animals. In comparison to the control diet, both sphingolipid diets significantly reduced ∑MUFA in PI in intestinal mucosa by 24% (the SM diet) and 26% (the GG diet).

5.3.3.6. Phosphatidylethanolamine (PE)

When dietary ganglioside was fed to animals, a higher level of PUFA was observed in 20:4n-6, 20:5n-3, 22:4n-6, and 22:6n-3 in PE compared to the control diet (Table V-7). While 24:0 increased, 24:1 decreased in animals fed the GG diet. Unlike the GG diet, the SM diet did not increase PUFA except for 20:5n-3. 16:0 and 24:1 decreased while 24:0 increased in animals fed the SM diet compared to control animals. Increased ∑PUFA in animals fed sphingolipid diets resulted in a decrease in the ∑SFA/∑PUFA ratio compared to the control diet in PE in intestinal mucosa.

5.4. DISCUSSION

The present data demonstrates that dietary ganglioside significantly alters the phospholipid profile in both total and individual phospholipid content as well as the fatty acid composition in developing intestine. The degree of changes in total and individual phospholipids and in the fatty acid composition in the intestine was different between two sphingolipid diets. For example, dietary ganglioside decreased total phospholipids and PC ($P<0.05$ and $P<0.0001$, respectively) (Figure V-1 and Table V-1). Feeding animals the SM diet showed a more significant alteration in individual phospholipids such as

Table V-5. Fatty acid composition of PS in intestinal mucosa fed the control or sphingolipid diets in rats[1]

Fatty acid	Control	SM	GG	Diet effect (P)
C 14:0	1.2± 0.6[b]	1.4± 0.4[b]	2.7± 0.9[a]	0.01
C 16:0	9.6± 1.3[ab]	8.8± 2.4[b]	11.7± 1.6[a]	0.05
C 18:0	59.3± 1.7	58.5± 3.0	54.6± 5.1	
C 18:1(9)	8.9± 0.6	9.0± 0.8	10.8± 3.0	
C 18:2(6)	6.5± 0.9	6.9± 1.2	6.2± 0.7	
C 18:3(3)	0.3± 0.1	0.4± 0.3	0.1± 0.2	
C 20:0	1.7± 0.5	2.0± 1.0	1.3± 0.3	
C 20:1(9)	0.4± 0.0	0.3± 0.2	0.3± 0.3	
C 20:2	0.3± 0.0	0.0± 0.1	0.1± 0.3	
C 20:3(6)	0.7± 0.1[a]	0.6± 0.1[a]	0.5± 0.1[b]	0.01
C 20:4(6)	3.2± 0.2[b]	3.7± 0.7[ab]	4.1± 0.5[a]	0.05
C 20:5(3)	0.2± 0.2[b]	1.1± 0.4[a]	1.2± 0.4[a]	0.0001
C 22:0	1.7± 0.3	1.9± 0.5	1.5± 0.2	
C 22:1(9)	0.5± 0.1	0.4± 0.2	0.6± 0.5	
C 22:2	0.4± 0.2[a]	0.1± 0.1[b]	0.1± 0.1[b]	0.01
C 22:4(6)	0.9± 0.1	0.7± 0.2	0.8± 0.5	
C 24:0	1.1± 0.4	1.9± 0.8	1.5± 0.4	
C 22:6(3)	1.5± 0.4	1.8± 0.4	1.5± 0.6	
C 24:1(9)	1.7± 0.2[a]	0.5± 0.4[b]	0.4± 0.3[b]	0.0001
ΣSFA[2]	74.5± 1.4	74.0± 2.0	73.0± 4.4	
ΣMUFA	11.5± 0.7	10.2± 1.1	12.0± 3.9	
ΣPUFA	14.0± 1.1	15.3± 2.2	14.7± 2.0	
ΣSFA/ΣPUFA	5.4± 0.5	5.0± 0.9	5.1± 0.8	

[1] Values are means ± SD of 6 rats. [2] SFA, MUFA and PUFA stand for saturated fatty acids, mono-unsaturated fatty acids and poly-unsaturated fatty acids, respectively.

Table V-6. Fatty acid composition of PI in intestinal mucosa fed the control or sphingolipid diets in rats[1]

Fatty acid	Control	SM	GG	Diet effect (P)
C 14:0	1.5± 0.3[b]	1.3± 0.6[b]	2.5± 1.1[a]	0.0001
C 16:0	13.4± 1.4	12.4± 2.3	13.8± 1.8	
C 18:0	37.8± 1.7	37.7± 1.9	38.0± 1.1	
C 18:1(9)	9.3± 0.3[a]	8.0± 1.1[b]	7.4± 0.7[b]	0.01
C 18:2(6)	7.2± 1.6	7.1± 0.8	5.8± 0.5	
C 18:3(3)	0.3± 0.1	0.1± 0.1	0.3± 0.4	
C 20:0	0.6± 0.2	0.7± 0.2	0.6± 0.3	
C 20:1(9)	0.3± 0.0	0.1± 0.1	0.2± 0.2	
C 20:2	0.5± 0.0[a]	0.1± 0.1[b]	0.2± 0.2[b]	0.01
C 20:3(6)	1.0± 0.2	0.7± 0.3	0.8± 0.1	
C 20:4(6)	22.3± 2.1	25.3± 1.0	22.6± 2.9	
C 20:5(3)	0.2± 0.0[c]	1.1± 0.2[b]	1.5± 0.3[a]	0.0001
C 22:0	1.0± 0.3	1.2± 0.4	1.3± 0.5	
C 22:1(9)	0.3± 0.1[a]	0.1± 0.1[b]	0.3± 0.1[a]	0.01
C 22:2	0.4± 0.2[a]	0.1± 0.2[b]	0.2± 0.2[ab]	0.05
C 22:4(6)	0.6± 0.2	0.4± 0.2	0.6± 0.2	
C 24:0	0.6± 0.2[b]	1.7± 0.8[a]	1.7± 0.3[a]	0.01
C 22:6(3)	1.3± 0.3	1.4± 0.6	1.7± 0.6	
C 24:1(9)	1.6± 0.2[a]	0.6± 0.2[b]	0.6± 0.3[b]	0.0001
ΣSFA[2]	54.7± 2.8	55.1± 1.8	57.9± 2.6	
ΣMUFA	11.5± 0.4[a]	8.8± 0.7[b]	8.5± 0.8[b]	0.0001
ΣPUFA	33.6± 2.8	36.2± 1.0	33.7± 2.8	
ΣSFA/ΣPUFA	1.7± 0.2	1.5± 0.1	1.7± 0.2	

[1] Values are means ± SD of 6 rats. [2] SFA, MUFA and PUFA stand for saturated fatty acids, mono-unsaturated fatty acids and poly-unsaturated fatty acids, respectively.

Table V-7. Fatty acid composition of PE in intestinal mucosa fed the control or sphingolipid diets in rats[1]

Fatty acid	Control	SM	GG	Diet effect (P)
C 14:0	0.5± 0.2	0.4± 0.2	0.6± 0.4	
C 16:0	7.3± 0.8^{a}	5.8± 1.3^{b}	6.4± 0.4^{ab}	0.05
C 18:0	38.6± 1.1	38.6± 1.2	37.0± 1.6	
C 18:1(9)	14.1± 1.2	13.2± 1.0	12.6± 1.1	
C 18:2(6)	15.0± 0.8	14.7± 0.9	13.9± 0.9	
C 18:3(3)	0.5± 0.3	0.3± 0.1	0.3± 0.3	
C 20:0	0.4± 0.1	0.5± 0.0	0.7± 0.4	
C 20:1(9)	0.4± 0.1	0.5± 0.1	0.5± 0.2	
C 20:2	0.2± 0.1	0.2± 0.1	0.2± 0.1	
C 20:3(6)	0.7± 0.1	0.7± 0.0	0.6± 0.2	
C 20:4(6)	16.1± 1.9^{b}	17.4± 1.3^{ab}	19.0± 0.9^{a}	0.01
C 20:5(3)	0.3± 0.2^{b}	0.7± 0.1^{a}	0.6± 0.2^{a}	0.01
C 22:0	0.3± 0.1^{b}	0.5± 0.1^{a}	0.4± 0.1^{ab}	0.05
C 22:1(9)	0.1± 0.1	0.2± 0.0	0.2± 0.1	
C 22:2	0.1± 0.1	0.1± 0.1	0.1± 0.1	
C 22:4(6)	1.2± 0.3^{b}	1.5± 0.2^{ab}	1.6± 0.2^{a}	0.05
C 24:0	0.1± 0.1^{b}	0.4± 0.1^{a}	0.4± 0.1^{a}	0.0001
C 22:6(3)	3.9± 0.4^{b}	4.4± 0.2^{b}	5.0± 0.8^{a}	0.01
C 24:1(9)	0.3± 0.2^{a}	0.1± 0.1^{b}	0.1± 0.0^{b}	0.01
ΣSFA[2]	47.1± 1.3	46.1± 1.1	45.5± 1.5	
ΣMUFA	15.0± 1.3	14.0± 1.1	13.4± 1.3	
ΣPUFA	37.9± 1.8^{b}	39.9± 0.9^{a}	41.2± 1.0^{a}	0.01
ΣSFA/ΣPUFA	1.3± 0.1^{a}	1.1± 0.1^{b}	1.1± 0.1^{b}	0.01

[1] Values are means ± SD of 6 rats. [2] SFA, MUFA and PUFA stand for saturated fatty acids, mono-unsaturated fatty acids and poly-unsaturated fatty acids, respectively.

increases in SM and PE and a decrease in PC ($P<0.05$, $P<0.01$, and $P<0.0001$, respectively), but not in total phospholipids (Table V-1). Animals fed the GG diet consumed much lower amount of sphingolipids from the diet compared to that of the SM diet (0.1% vs 1%). This data suggests a hypothesis that the effect of ganglioside on reduction of total phospholipid content may be due to NANA molecules. In this regard, a previous animal study may support this assumption. Long term feeding of 1% sphingolipids (cerebroside plus SM) did not affect the phospholipid content in the rat liver (43). It is thus suggested that gangliosides, but not sphingolipids without NANA or sugars, have a unique role in reducing total phospholipid content in the intestinal mucosa. A previous study shows that total phospholipid content in the intestine decreases during development from the neonate to adult rat (44). Further study is needed to determine if dietary gangliosides influence developmental maturity of membrane permeability or morphology in the intestine.

In individual phospholipids, both dietary sphingolipids showed significant increases in PUFA including 20:4n-6, 20:5n-3, 22:5n-3, and 22:6n-3. The effect of both sphingolipids on increases in PUFA may be indirectly explained by ganglioside and cholesterol content in the intestinal mucosa. Our previous study demonstrated that these two sphingolipids decreased total cholesterol content and dietary ganglioside increased total gangliosides in the intestinal mucosa (45). As enterocytes have desaturases (46), cholesterol reduction in the intestine may stimulate the activity of Δ-5 and Δ-6 desaturases in fatty acid desaturation (47). It is also known that increased ganglioside or decreased cholesterol content attenuates PLA2 activity, leading to decreased release of PUFA from the membrane (48,49). Higher levels of PUFA in phospholipids observed in animals fed the GG diet may be explained because dietary ganglioside increased ganglioside and decreased cholesterol, whereas dietary sphingomyelin only decreased cholesterol. These results suggest that dietary sphingolipids may result in release of lower amounts of arachidonic acids during inflammation. In this regard, previous studies demonstrate an inhibitory effect of ganglioside GM1 and GM3 on PLA2 activity (49,50).

Increased PUFA in intestinal phospholipids as a result of consuming gangliosides raises a question of whether these PUFA may serve a particular enterocyte function. Since animals fed a diet high in PUFA increase glucose uptake and decrease leucine

uptake in the intestine (51), increased intestinal PUFA in the present study may be associated with alteration of active and passive nutrient transport systems. Enhanced PUFA content in the intestine may increase the mucus thickness by influencing musin glycosylation (52). Increased n-6 and n-3 PUFA increases duodenal calcium uptake (53,54). These fatty acid changes also alter Ca^{2+}-ATPase and Na^{+}/K^{+}-ATPase in the intestine (55,56). Increased 20:4n-6 in PC and PE is known to decrease lactase activity in the suckling rat intestine (57). The enzyme activity becomes weaker from newborn to adult rats (58). These findings may also suggest that dietary ganglioside influences the maturity of enterocytes because animals fed the GG diet showed high level of 20:4n-6 in PC and PE (Table V-4 and V-7).

In summary, the present study demonstrates that dietary ganglioside alters the content and composition of phospholipids as well as the fatty acid composition of phospholipids in the developing gut. Further studies are needed to determine if dietary ganglioside influences developmental maturity and gut-associated functional properties in developing intestines.

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CHAPTER VI

DIETARY GANGLIOSIDE INCREASES CONTENT OF ETHER PHOSPHOLIPIDS CONTAINING 20:4n-6 AND 22:6n-3 IN THE RAT INTESTINE

6.1. INTRODUCTION

Gangliosides, sialic acid-containing glycosphingolipids, act as a receptor for *E.coli* and *Cholera* toxins (1) and stimulator of the immune system in the intestine (2). Rat intestine has 20%~30% of the total lipids as glycosphingolipids, one third of which are gangliosides (3). Change occurs in the composition and molecular structure of gangliosides during intestinal development (4, 5). GM3 is the major ganglioside in rat intestine (5) and is localized at the brush border membrane while GD3 is present at the basolateral membrane (6). These findings imply that intestinal function may be influenced by the presence and composition of constituent sphingolipids (SPL).

Exogenous ganglioside and sphingomyelin is hydrolyzed by enterocyte membrane-bound enzymes such as sialidase, sphingomyelinase and/or ceramidase (7-10). When rats were fed radiolabelled sphingosine, ceramide or sphingomyelin, about 10~55% of radioactivity was found in the rat intestine and 30~60% is found in lymph lipids after 24 h (9). Metabolites transported into enterocytes are reutilized in synthesis of gangliosides or sphingomyelin or both (7, 8). These studies indicate that dietary SPL are digested, metabolised and transported into other tissues.

Several studies suggest a possible interaction between SPL and phospholipids (7-9, 11). Sphingosine-1-phosphate, a metabolic derivatives of SPL, is metabolized into ethanolamine phosphate and hexadecanal, both prerequisite materials for phospholipid synthesis (7, 8). In animal studies, dietary [3-^{3}H]sphingosine-sphingomyelin is absorbed of which 70% is fatty acid and glyceride (9). An appreciable amount of sphingosine is incorporated into hepatocyte phospholipids in the ether phospholipid (EPL) form when radiolabelled [^{3}H]sphingosine-GM1 was intraperitoneally injected into mice (11).

EPL have an ester linkage at the sn-2 position, and an ether linkage, either to an alkyl or alkenyl group, at the sn-1 position (12). EPL tend to be enriched in mammalian neuronal and intestinal cells (12, 13). One type of EPL known as plasmalogen, 1-

O-alkenyl-2-acyl-glycero-phospholipids, accounts for about 6~12% of EPG in rat intestinal mucosa (13, 14). High content of EPL may contribute to maintenance of cell integrity and function (15-24) such as permeability (16) fluidity (21) and endogenous antioxidant for membrane peroxidation (25-27). EPL also induce cell apoptosis (15), cytotoxicity (17, 18), and antitumor activity (19, 22), that could have potential in anti-cancer applications. These cellular functions of EPL seem to be dependent to membrane cholesterol content (28). For example, cholesterol reduction in the membrane causes increased EPL uptake into the membrane (28, 29) and increases activity of Δ-5 and Δ-6 desaturase enzymes (30).

Our previous work (31) demonstrates that dietary ganglioside increases total content of gangliosides and decreases cholesterol content in developing rat intestine. Thus it was logical to hypothesize that dietary ganglioside will increase the content of poly-unsaturated fatty acids (PUFA) in EPL in the intestine by increasing derivatives of SPL and decreasing cholesterol content. Suckling babies ingest about ~35~170 mg SPL from mothers' milk per day (32). No clear information is available to explain the metabolic fate of SPL to EPL in the developing intestine. Radiolabelled studies (7-9) showing a possible mechanism to convert sphingolipids to EPL in vivo do not explain if dietary ganglioside can be absorbed and then used for EPL synthesis because in vitro or radiolabelled studies are not representative of physiological condition. Thus, the objective for this study was to determine if dietary ganglioside increases total membrane EPL content. This study also examined whether increased EPL content is accompanied with higher PUFA in EPL. Sphingomyelin and ganglioside have the same ceramide molecule anchored in the cell membrane, but attached to a different head group. Thus, sphingomyelin was used as a second control to compare bioavailability with gangliosides. Using rats, the present study demonstrates that dietary ganglioside increases total content and composition of EPL containing PUFA in the developing intestine.

6.2. MATERIALS AND METHODS

6.2.1. Animals and diets

The protocol for this study was approved by the Animal Care Committee at the University of Alberta, Canada. Male Sprague-Dawley rats (18-day old, 40 ± 4.5 g) were

housed in polypropylene cages and maintained at a constant room temperature of 23°C and a 12 h light/dark cycle for 2 weeks. Animals had free access to water and were randomized to be fed one of three semi-purified diets containing 20% (w/w) fat (Table VI-1). The composition of basal diet is reported elsewhere (33). Two experimental diets were formulated by adding either sphingomyelin (1.0% w/w of total fat, Sigma, MO, USA; SM diet) or ganglioside (0.1% w/w of total fat, New Zealand Dairy, New Zealand; GG diet) to the control diet. The ganglioside fraction in the ganglioside enriched diet contained about 80% (w/w) GD3. GD1b, GM3 and other gangliosides were 9, 5 and 6% w/w, respectively. The fatty acid composition of experimental diets was quantitatively analyzed by gas liquid chromatography (GLC, Varian Vista 3400CX). The fatty acid composition was consistent among the three diets: 18:1n-9 (50%), 18:2n-6 (20%), 16:0 (16%), 18:0 (8%), 18:3n-3 (2.8%) and other fatty acids (3.2%). The control diet and experimental diets provided an n-6 to n-3 ratio of 7:1. Cholesterol content was negligible (<0.002% w/w of total fat). Body weight and food intake was measured every other day throughout the experiment.

Table VI-1. Composition of experimental diets

	Control	SM	GG
Basal diet[1] (g/100g)	80.0	80.0	80.0
Triglyceride[2]	20.0 (100)[4]	19.8 (99.0)	19.9 (98.6)
Sphingomyelin	-	0.2 (1.0)	tr[3]
Ganglioside	-	-	0.02 (0.1)
Phospholipid	-	-	0.05 (0.25)
Cholesterol	-	-	tr[3] (0.002)

[1] The composition of the basal diet was described by Clandinin and Yamashiro (33).
[2] The fatty acid composition of the triglyceride fed was similar to that of an infant formula (34). [3] tr represents trace amount. [4] Values in parenthesis represent the percentage of total fat.

6.2.2. Collection of intestinal mucosa

After anesthetizing animals with halothane, the small intestine (jejunum to ileum) was excised. The intestine was washed with 0.9 % cold saline solution to remove visible mucus and debris, opened longitudinally, and moisture was carefully removed. Intestinal mucosa was scraped off with a glass slide on an ice cold glass plate. All samples were weighed and kept in a –70 °C freezer until used.

6.2.3. Lipid extraction and phospholipid separation

Total lipid was extracted by using the Folch method (35). For extracting total lipid, the intestinal mucosa was washed twice with Folch lower phase solution (chloroform/methanol/ water, 86:14:1, by vol). The lower phase lipid was pooled, dried and dissolved in chloroform/methanol (2:1, v/v). Extracted lipid was applied onto pre-coated silica gel "H" thin layer chromatography (TLC) plates (Analtech, Newark, DE) and developed in the solvent (chloroform/methanol/2-propanol/0.25% KCl/triethylamine, 45:13.5:37.5:9:27, by vol) to separate individual phospholipid classes. After spraying with 0.1 % ANSA (anilino naphthalene sulfonic acid) and identifying CPG and EPG bands under UV light, the two bands were scraped into test tubes.

6.2.4. Fatty acid composition and quantification

Separation and purification of three subclasses of PC and PE were prepared (36-38). Solid phases containing CPG and EPG were eluted with 5 mL of chloroform/methanol (2:1, v/v) and dried under N_2. Phospholipids were dephosphorylated with phospholipase C (*B. cereus*, Sigma Chemical Co., St. Louis, MO) by the method of Bernett et al (39) to provide for alkenylacyl, alkylacyl, and diacyl fractions from each phospholipid. After extraction of diradylglycerols with diethyl ether, lipids were acetylated in 0.1 mL of pyridine and 0.5 mL of acetic anhydride at 80°C for 1 h. The acetylated derivatives of 3 subclasses of PC and PE were applied onto a silica gel high performance TLC plate (HPTLC, Whatman Inc, Clifton, NJ) and developed in petroleum ether: diethyl ether: acetic acid, (90:10:1, by vol) to a migration distance of 10 Cm from the solvent line, followed by a second development in toluene (36). Three subclasses of the CPG and EPG fractions scraped from the plate were then methylated in

3N-methanolic-HCl (Supelco, PA, USA) for 16 h at 70°C with a known amount of heptadecanoic acid as an internal standard. The fatty acid composition of each of the six fractions was analyzed by GLC equipped with a flame ionization detector and BP-20 fused capillary column (SGE, Australia). The flow rate of helium gas was 1.6 mL/min and the oven, injector and detector temperatures were 200°C, 250°C, and 250°C, respectively. To compare the molecular percentage of diacyl phospholipid with ether phospholipid, total fatty acid amount of the diacyl subclass was divided by two because only one fatty acid is derived from ether phospholipid (37, 38). To examine the recovery ratio of three subclasses from the initial PC and PE fraction through purification and separation processes, ten percent of PC and PE fractions was used for analyzing the total content of fatty acids in PC and PE. The remaining ninety percent of each fraction was used for EPL analysis. The average recovery ratio of three subclasses of PC and PE was 75% (data not shown).

6.2.5. Statistical analysis

The values were shown as means ± standard deviation from eight animals, with a few exceptions indicated. Significant differences between the control group and experimental groups were determined by one-way analysis of variance (ANOVA) with SAS (SAS Institute Inc, Version 8.2, NC, USA). Effects of diet treatment were determined by a Duncan multiple range test at significance levels of $P<0.05$, $P<0.01$, $P<0.001$ or $P<0.0001$.

6.3. RESULTS

6.3.1. Animal growth and intestinal mucosa

Initial body weight of animals and weight after two weeks of diet treatment was not significantly different between experimental and control groups. Intestinal mucosal weight and length was not affected by dietary treatment. Food consumption was also not influenced by diet treatment (data not shown).

6.3.2. Fatty acid content corresponding to alkenylacyl, alkylacyl and diacyl-GPC and GPE

Animals fed the GG diet significantly increased the content of total fatty acids in alkenylacyl-GPC, alkenylacyl-GPE and alkylacyl-GPE in comparison with control animals (56%, 77% and 54%, respectively; Table VI-2). In animals fed dietary ganglioside, a significant decrease in diacyl-GPC occurred, whereas no change was observed in diacyl-GPE. Animals fed the SM diet exhibited a similar increase in fatty acid content in EPL corresponding to alkylacyl-GPE, but no change occurred in the other subclasses of GPC and GPE. The GG diet produced significant increase in the total fatty acid content of alkenylacyl and alkylacyl together by 36% and 66% respectively, in CPG and EPG phospholipids (Table VI-2). Feeding animals the SM diet increased total EPL in EPG by 42% compared to control animals.

Table VI-2. Fatty acid content of alkenylacyl-, alkylacyl- and diacyl-GPC and GPE from intestinal mucosa of animals fed experimental diets[1]

Subclass (µg/g tissue)	Control	SM	GG	Diet Effect (P)
Total EPL-GPC[2]	31.8 ± 6.7[b]	37.8 ± 8.7[ab]	43.3 ± 8.2[a]	0.05
Alkenylacyl-GPC	14.0 ± 3.6[b]	17.0 ± 3.0[ab]	21.8 ± 7.2[a]	0.05
Alkylacyl-GPC	17.8 ± 3.9	20.8 ± 7.5	21.5 ± 4.8	
Diacyl-GPC	1860 ± 340[a]	1610 ± 470[ab]	1220 ± 310[b]	0.01
Total EPL-GPE	68.1 ± 7.9[c]	97.1 ± 15[b]	113 ± 20[a]	0.001
Alkenylacyl-GPE	34.4 ± 7.7[b]	45.2 ± 15[b]	60.9 ± 12[a]	0.001
Alkylacyl-GPE	33.7 ± 4.4[b]	51.9 ± 11[a]	51.8 ± 11[a]	0.001
Diacyl-GPE	774 ± 99	731 ± 290	801 ± 170	

[1] Means ± SD from 7, 8, and 7 animals, for the Control, SM and GG group, respectively.
[2] Fatty acid content of total EPL (alkenylacyl and alkylacyl together) in CPG or EPG.

6.3.3. Molecular percentage of alkenylacyl, alkylacyl and diacyl-GPC and GPE

Animals fed the GG diet exhibited higher levels of alkenylacyl-GPC (3.3% versus 1.6%) and alkylacyl-GPC (3.2% *vs* 2.1%) with correspondingly lower levels of diacyl-GPC (93.5% *vs* 96.3%), compared to animals fed the control diet (Table VI-3). No effect of the SM diet was observed on the relative composition of CPG in the mucosa. Feeding animals the GG diet achieved higher molecular levels of alkenylacyl-GPE (12.5% versus 7.4%) and alkylacyl-GPE (10.9% *vs* 7.3%) with correspondingly lower molecular levels of diacyl-GPE (76.6% *vs* 85.3%), compared to animals fed the control diet. Animals fed the SM diet showed a relative increase in alkenylacyl-GPE, but the effect was less pronounced than in those fed the GG diet. There was no effect of the SM diet on alkylacyl-GPE. Relative to molecular percentage in 3 alkenylacyl-, alkylacyl- and diacyl classes, animals fed the GG diet exhibited a 76% increase in CPG and 59% increase in total alkenylacyl- and alkylacyl-EPL (Table VI-3). Feeding the SM diet resulted in a molecular increase in EPG, but not in CPG.

Table VI-3. Molecular percentage of alkenylacyl-, alkylacyl- and diacyl-GPC and GPE from intestinal mucosa of animals fed experimental diets[1]

Subclass (%, w/w)	Control	SM	GG	Diet Effect (P<)
Total-EPL-GPC[2]	3.7 ±0.8[b]	4.8 ± 1.1[b]	6.5 ± 1.8[a]	0.01
Alkenylacyl-GPC	1.6 ±0.4[b]	2.2 ± 0.8[ab]	3.3 ± 1.3[a]	0.05
Alkylacyl-GPC	2.1 ±0.5[b]	2.6 ± 0.6[ab]	3.2 ± 0.8[a]	0.05
Diacyl-GPC	96.3 ±0.8[a]	95.2 ± 1.1[a]	93.5 ± 1.8[b]	0.01
Total-EPL-GPE	14.7 ±1.6[b]	20.4 ± 5.4[a]	23.4 ± 3.2[a]	0.001
Alkenylacyl-GPE	7.4 ±1.5[c]	10.2 ± 2.1[b]	12.5 ± 1.4[a]	0.0001
Alkylacyl-GPE	7.3 ±1.1[b]	10.2 ± 3.7[ab]	10.9 ± 2.5[a]	0.05
Diacyl-GPE	85.3 ±1.6[a]	79.6 ± 5.4[b]	76.6 ± 3.2[b]	0.01

[1] Means ± SD from 7, 8, and 7 animals, for the Control, SM and GG group, respectively.
[2] Percent of total EPL (alkenylacyl and alkylacyl together) relative to total phospholipids in CPG or EPG.

6.3.4. Fatty acid composition of alkenylacyl-, alkylacyl- and diacyl-GPC

In comparison with control animals, animals fed the GG diet did not show significant change in the fatty acid composition of alkenylacyl-GPC (Table VI-4). Animals fed the GG diet showed an increase in 20:4n-6 compared to animals fed the SM diet. Decrease in alkylacyl-GPC content of 18:0, 24:0 and 24:1 occurred for animals fed the GG diet as well as a distinct increase by 2.5-fold in 22:6n-3 ($P<0.001$) compared to control animals. The SM diet produced a decrease in 18:0 and an increase in 22:6n-3 content. In diacyl-GPC, higher levels of 16:0 and 18:0 and lower levels of 18:1n-9 and 18:2n-6 were observed in animals fed the GG diet compared to control animals. Similar trends in 16:0, 18:1n-9 and 18:2n-6 were observed for animals fed the SM diet.

6.3.5. Fatty acid composition of alkenylacyl-, alkylacyl- and diacyl-GPE

The fatty acid composition of alkenylacyl-, alkylacyl-, and diacyl-GPE in intestinal mucosa of animals fed experimental diets is illustrated (Table VI-5). Animals fed dietary ganglioside showed higher levels of 22:4n-6 in alkenylacyl-GPE (100% increase, $P<0.001$) and 22:6n-3 (71% increase, $P<0.001$), and lower levels of 16:0 and 18:0 compared to feeding the control diet. Animals receiving the SM diet exhibited a similar change in 22:4n-6, 22:6n-3 and 16:0 fatty acid content, but the effect was smaller than observed for animals fed the GG diet. In alkylacyl-GPE, higher content of 20:4n-6, 22:4n-6, and 22:6n-3 (36%, 87% and 77% increases, respectively) was observed in animals fed the GG diet with a considerable reduction in saturated fatty acids, specifically 16:0, 18:0 and 24:0 relative to animals fed the control diet. Dietary ganglioside increased the content of 20:4n-6 by 36% in diacyl-GPE, relative to control diet.

6.3.6. Total SFA, MUFA and PUFA content of alkenylacyl-, alkylacyl- and diacyl-GPC

The fatty acid content of SFA, MUFA and PUFA in alkenylacyl-, alkylacyl-, and diacyl-GPC is shown (Table VI-4). Feeding animals the GG diet increased total PUFA content in alkylacyl-GPC by 63% compared to animals fed the control diet. No changes occurred in MUFA and SFA for this lipid subclass. Animals fed the GG diet had lower content of PUFA and MUFA and increased content of SFA in diacyl-GPC than observed

Table VI-4. Fatty acid composition of alkenylacyl-, alkylacyl- and diacyl-GPC in intestinal mucosa[1]

	Alkenylacyl-GPC			Alkylacyl-GPC			Diacyl-GPC		
	Control	SM	GG	Control	SM	GG	Control	SM	GG
C 14:0	6.2 ± 1.6	8.1 ± 5.4	5.0 ± 1.3	5.4 ± 2.1	6.1 ± 2.5	6.4 ± 2.5	0.8 ± 0.2	1.1 ± 0.3	0.9 ± 0.2
C 14:1	1.9 ± 1.5^{a}	0.5 ± 0.3^{b}	0.4 ± 0.6^{b}	1.0 ± 0.7	0.3 ± 0.4	0.5 ± 0.5	-	-	-
C 16:0	33.7 ± 5.8	32.2 ± 8.6	32.3 ± 8.2	34.3 ± 7.3	33.3 ± 6.1	33.0 ± 4.3	21.0 ± 1.7^{b}	24.4 ± 2.2^{a}	24.3 ± 2.5^{a†}
C 16:1(7)	1.4 ± 1.6	0.8 ± 0.5	1.1 ± 1.6	0.4 ± 0.6	0.7 ± 0.6	0.4 ± 0.4	0.4 ± 0.0	0.3 ± 0.2	0.2 ± 0.1
C 18:0	25.5 ± 3.7	23.2 ± 5.9	22.0 ± 6.7	25.8 ± 3.6^{a}	17.6 ± 5.7^{b}	19.0 ± 4.6^{b†}	22.7 ± 1.1^{b}	24.8 ± 3.4^{b}	27.7 ± 3.2^{a†}
C 18:1(9)	8.8 ± 4.3	8.5 ± 4.3	5.9 ± 2.3	8.7 ± 3.1	7.7 ± 1.5	7.4 ± 1.3	11.6 ± 0.8^{a}	10.5 ± 0.8^{b}	9.2 ± 1.1^{c¶}
C 18:2(6)	3.2 ± 1.8	3.2 ± 2.1	2.4 ± 1.6	5.3 ± 4.1	5.5 ± 1.8	5.5 ± 2.9	30.0 ± 1.6^{a}	26.6 ± 3.1^{b}	23.5 ± 3.4^{c‡}
C 18:3(6)	0.5 ± 0.4	1.0 ± 1.2	0.7 ± 0.8	0.8 ± 0.6	0.7 ± 0.4	0.6 ± 0.7	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.1
C 18:3(3)	0.5 ± 0.6	0.3 ± 0.2	0.6 ± 0.6	0.5 ± 0.9	0.1 ± 0.2	0.4 ± 0.3	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
C 20:0	1.2 ± 1.2	1.0 ± 0.4	1.9 ± 1.7	1.0 ± 0.2	0.8 ± 0.4	0.9 ± 0.6	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.1
C 20:1	0.3 ± 0.4	0.6 ± 0.3	1.0 ± 0.7	0.4 ± 0.3	0.4 ± 0.4	0.6 ± 1.0	0.5 ± 0.1	0.4 ± 0.0	0.4 ± 0.1
C 20:2	0.0 ± 0.1	0.1 ± 0.1	0.7 ± 1.1	0.1 ± 0.3	0.1 ± 0.2	2.6 ± 3.0	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1
C 20:3(6)	0.3 ± 0.4	0.6 ± 0.7	0.4 ± 0.5	0.5 ± 0.6^{b}	1.3 ± 1.3^{a}	0.0 ± 0.1^{b†}	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
C 20:4(6)	1.8 ± 0.6^{ab}	1.1 ± 0.5^{b}	3.1 ± 2.1^{a}	5.7 ± 3.7	9.5 ± 4.8	9.3 ± 6.7	9.4 ± 0.4	8.8 ± 2.8	10.2 ± 1.5
C 20:3(3)	0.3 ± 0.4	0.9 ± 0.8	0.7 ± 0.8	0.4 ± 0.4	0.4 ± 0.4	0.3 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
C 20:5(3)	1.5 ± 0.9	0.6 ± 0.6	2.2 ± 2.0	0.7 ± 0.6	0.8 ± 0.5	1.0 ± 0.5	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1
C 22:0	0.5 ± 0.4^{b}	2.6 ± 1.5^{a}	2.2 ± 2.1^{ab}	1.3 ± 0.8	2.2 ± 1.3	1.8 ± 1.0	0.2 ± 0.0	0.2 ± 0.1	0.3 ± 0.2
C 22:1(9)	1.3 ± 1.0	1.5 ± 2.3	1.5 ± 1.6	0.1 ± 0.2^{b}	1.2 ± 1.0^{ab}	1.7 ± 1.5^{a}	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
C 22:2(6)	2.1 ± 1.1^{ab}	1.5 ± 0.9^{b}	4.0 ± 2.9^{a}	0.0 ± 0.0^{b}	2.9 ± 2.6^{a}	1.2 ± 1.4^{ab†}	0.0 ± 0.0	0.1 ± 0.2	0.1 ± 0.1
C 22:4(6)	0.1 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.4	0.7 ± 0.9	0.6 ± 0.8	0.1 ± 0.1	0.1 ± 0.2	0.1 ± 0.0
C 24:0	2.4 ± 1.5	3.3 ± 1.5	3.2 ± 2.2	1.8 ± 1.0^{a}	1.8 ± 1.0^{a}	0.6 ± 0.8^{b}	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.1
C 22:6(3)	1.2 ± 1.1	0.9 ± 1.1	tr[2]	1.2 ± 1.0^{b}	2.7 ± 0.9^{a}	4.2 ± 1.8^{a‡}	1.4 ± 0.2	1.1 ± 0.4	1.3 ± 0.2
C 24:1(9)	5.3 ± 2.9	7.5 ± 5.4	8.8 ± 3.6	4.0 ± 1.8^{a}	3.0 ± 0.7^{ab}	1.8 ± 1.4^{b}	0.1 ± 0.0	0.1 ± 0.1	0.2 ± 0.1
Total	100	100	100	100	100	100	100	100	100
SFA[3]	69.5 ± 7.3	70.4 ± 10.9	66.6 ± 6.6	69.6 ± 10.6	61.8 ± 9.3	61.7 ± 7.8	45.2 ± 2.3^{b}	50.7 ± 5.5^{a}	53.6 ± 5.8^{a†}
MUFA	19.1 ± 4.8	19.4 ± 7.3	18.7 ± 2.6	14.6 ± 2.5	13.2 ± 1.9	12.4 ± 2.2	12.8 ± 0.9^{a}	11.5 ± 0.9^{b}	10.2 ± 1.1^{c¶}
PUFA	11.4 ± 3.6	10.2 ± 5.2	14.8 ± 4.9	15.8 ± 8.9^{b}	25.0 ± 7.6^{a}	25.9 ± 6.6^{a}	42.0 ± 1.7^{a}	37.7 ± 5.7^{ab}	36.2 ± 4.8^{b}

[1] Means ± SD (% w/w) in 3 subclasses from 7, 8 and 7 animals, for the control, SM and GG group, respectively. Within a row, values with different superscript letters are significantly different at $P < 0.05$. Superscript letters with $^{\dagger, \ddagger, \text{and } \P}$ are significantly different at $P<0.01$, $P<0.001$, and $P<0.0001$, respectively. [2] tr represents trace amount. [3] SFA, MUFA and PUFA represent saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids, respectively.

Table VI-5. Fatty acid composition of alkenylacyl-, alkylacyl- and diacyl-GPE in intestinal mucosa[1]

	Alkenylacyl-GPE			Alkylacyl-GPE			Diacyl-GPE		
	Control	SM	GG	Control	SM	GG	Control	SM	GG
C 14:0	3.9 ± 1.3	3.1 ± 1.1	3.1 ± 1.2	4.4 ± 1.5	3.0 ± 1.9	2.5 ± 1.5	0.7 ± 0.4	0.4 ± 0.1	0.5 ± 0.2
C 14:1	0.7 ± 0.3	0.9 ± 1.0	0.5 ± 0.4	0.7 ± 0.6^{a}	0.2 ± 0.2^{b}	0.2 ± 0.1^{b}	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
C 16:0	18.9 ± 3.6^{a}	15.4 ± 1.7^{b}	14.2 ± 2.3^{b†}	18.7 ± 5.4^{a}	12.8 ± 3.2^{b}	11.4 ± 1.9^{b†}	9.8 ± 2.5	8.5 ± 1.8	8.1 ± 1.9
C 16:1(7)	2.1 ± 1.3	2.5 ± 0.7	2.3 ± 0.6	0.6 ± 0.5	0.3 ± 0.2	0.3 ± 0.3	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0
C 18:0	17.5 ± 4.7^{a}	14.4 ± 4.1^{ab}	11.6 ± 2.7^{b}	14.8 ± 3.2^{a}	11.3 ± 4.0^{b}	9.8 ± 2.1^{b}	47.0 ± 8.6	43.5 ± 7.4	42.6 ± 4.3
C 18:1(9)	8.9 ± 2.0	8.2 ± 1.2	7.3 ± 0.9	12.0 ± 3.1	11.7 ± 1.9	9.2 ± 1.3	12.2 ± 2.9	13.1 ± 2.1	12.8 ± 1.5
C 18:2(6)	4.0 ± 1.3	4.6 ± 0.5	4.1 ± 0.7	5.4 ± 1.0	6.4 ± 0.6	5.6 ± 0.8	11.8 ± 3.3	14.7 ± 3.0	14.2 ± 1.8
C 18:3(6)	0.6 ± 0.3^{a}	0.6 ± 0.1^{a}	0.3 ± 0.1^{b}	0.5 ± 0.4	0.5 ± 0.3	0.4 ± 0.2	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.0
C 18:3(3)	2.2 ± 1.2	2.1 ± 0.9	2.1 ± 1.0	0.5 ± 0.3	0.6 ± 0.3	0.6 ± 0.2	0.3 ± 0.5	0.2 ± 0.1	0.1 ± 0.0
C 20:0	0.6 ± 0.2	0.5 ± 0.2	0.5 ± 0.2	1.6 ± 1.2	1.1 ± 0.2	1.1 ± 0.1	0.5 ± 0.2	0.5 ± 0.1	0.3 ± 0.3
C 20:1	1.0 ± 0.8	1.0 ± 0.2	0.8 ± 0.1	2.1 ± 1.2	2.1 ± 0.4	2.1 ± 0.5	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
C 20:2	1.2 ± 1.4	0.5 ± 0.7	1.1 ± 0.9	0.4 ± 0.3	0.5 ± 0.3	0.7 ± 0.3	0.4 ± 0.6	0.2 ± 0.1	0.1 ± 0.0
C 20:3(6)	0.8 ± 0.6	0.9 ± 0.3	0.9 ± 0.2	1.0 ± 0.5	1.2 ± 0.6	1.6 ± 0.5	0.4 ± 0.2	0.5 ± 0.2	0.4 ± 0.3
C 20:4(6)	16.8 ± 5.7	21.3 ± 3.9	23.1 ± 4.9	18.5 ± 6.1^{b}	22.0 ± 4.6^{ab}	25.1 ± 2.6^{a}	11.5 ± 3.6^{b}	14.0 ± 3.5^{ab}	15.7 ± 2.6^{a†}
C 20:3(3)	0.5 ± 0.2^{a}	0.3 ± 0.2^{ab}	0.2 ± 0.1^{b}	0.4 ± 0.2	0.4 ± 0.2	0.3 ± 0.2	0.1 ± 0.1	0.1 ± 0.1	0.0 ± 0.0
C 20:5(3)	0.6 ± 0.3	0.4 ± 0.3	0.5 ± 0.2	0.8 ± 0.3	0.7 ± 0.4	0.6 ± 0.3	0.3 ± 0.1	0.3 ± 0.2	0.2 ± 0.2
C 22:0	1.3 ± 0.7	1.0 ± 0.6	0.9 ± 0.2	0.7 ± 0.6	0.7 ± 0.5	1.2 ± 0.9	0.5 ± 0.2	0.3 ± 0.1	0.4 ± 0.2
C 22:1(9)	0.4 ± 0.4	0.4 ± 0.4	0.8 ± 0.8	0.5 ± 0.5	1.1 ± 0.9	1.0 ± 1.1	0.3 ± 0.4	0.2 ± 0.1	0.2 ± 0.1
C 22:2(6)	1.2 ± 1.5	0.5 ± 0.2	0.7 ± 0.3	1.5 ± 0.9	1.6 ± 1.1	1.2 ± 0.5	0.4 ± 0.3^{a}	0.0 ± 0.0^{b}	0.0 ± 0.1^{b‡}
C 22:4(6)	4.9 ± 2.8^{b}	7.8 ± 1.6^{a}	9.9 ± 2.1^{a‡}	5.8 ± 2.3^{b}	8.3 ± 2.9^{ab}	10.9 ± 2.2^{a†}	0.3 ± 0.2	0.4 ± 0.2	0.6 ± 0.5
C 24:0	2.3 ± 1.9	1.4 ± 0.7	0.9 ± 0.5	1.4 ± 0.4^{a}	1.6 ± 0.7^{a}	0.7 ± 0.5^{b}	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.2
C 22:6(3)	7.3 ± 2.6^{c}	10.1 ± 0.9^{b}	12.7 ± 1.8^{a‡}	6.3 ± 1.9^{b}	9.2 ± 2.3^{a}	11.2 ± 1.2^{a‡}	2.0 ± 1.0	2.3 ± 1.1	2.7 ± 0.5
C 24:1(9)	2.5 ± 1.4	2.3 ± 1.5	1.5 ± 1.1	1.3 ± 0.7	2.6 ± 1.6	2.4 ± 2.3	0.6 ± 0.5	0.3 ± 0.1	0.2 ± 0.1
Total	100	100	100	100	100	100	100	100	100
SFA[2]	44.7 ± 9.7^{a}	35.7 ± 5.9^{b}	31.2 ± 5.5^{b†}	41.6 ± 9.8^{a}	30.2 ± 7.1^{b}	26.7 ± 4.4^{b†}	58.6 ± 9.8	53.3 ± 8.8	50.4 ± 4.9
MUFA	15.7 ± 4.2	15.2 ± 2.5	13.2 ± 2.1	17.2 ± 3.0	17.9 ± 2.8	15.2 ± 2.4	13.6 ± 2.8	13.9 ± 2.1	13.4 ± 1.1
PUFA	39.4 ± 11.3^{b}	49.1 ± 5.4^{a}	55.6 ± 7.4^{a†}	41.2 ± 11.5^{b}	51.9 ± 9.4^{a}	58.1 ± 4.2^{a†}	27.8 ± 7.4^{b}	32.8 ± 7.0^{ab}	36.2 ± 4.2^{a}

[1] Means ± SD (% w/w) in 3 subclasses from 7, 8 and 7 animals, for the control, SM and GG group, respectively. Within a row, values with different superscript letters are significantly different at $P< 0.05$. Superscript letters with $^{\dagger, \ddagger, \text{and } \P}$ are significantly different at $P<0.01$, $P<0.001$, and $P<0.0001$, respectively. [2] SFA, MUFA and PUFA represent saturated fatty acids, mono unsaturated fatty acids and poly unsaturated fatty acids, respectively.

for control animals. For animals fed the SM diet, a higher level of PUFA in alkylacyl-GPC was found. These animals also exhibited lower MUFA and higher SFA contents in diacyl-GPC compared to control animals.

6.3.7. Total SFA, MUFA and PUFA content of alkenylacyl-, alkylacyl- and diacyl-GPE

Animals fed the GG diet exhibited increased content of PUFA in the three EPG lipid subclasses compared to control animals, (increase of 41% in alkenylacyl, 41% in alkylacyl and 30% in diacyl-GPE; Table VI-5). The increase in PUFA content was accompanied with a decrease in SFA content in alkenylacyl- and alkylacyl-GPE. No change was observed in MUFA in alkenylacyl-, alkylacyl- or diacyl-phospholipids. Feeding of the SM diet also resulted in increased content of PUFA and decreased content of SFA in alkenylacyl-GPE and alkylacyl-GPE compared to controls. No effect of the SM diet was detected in diacyl-GPE.

6.3.8. Ratio of SFA/MUFA, and SFA/PUFA in alkenylacyl-, alkylacyl- and diacyl-GPC and GPE

As PUFA levels in alkylacyl-GPC increased in animals fed the GG diet, there was a concomitant decrease in the ratio of SFA to PUFA in alkylacyl-GPC (2.6 *vs* 6.8, $P<0.05$; Figure VI-1). In contrast, in diacyl-GPC fractions, animals fed the GG diet exhibited an increase in the ratio of SFA to MUFA (5.3 *vs* 3.6, $P<0.001$) and SFA to PUFA (1.5 *vs* 1.1, $P<0.05$).

Changes in relative amounts of SFA, MUFA, and PUFA in the three subclasses of EPG are illustrated (Figure VI-2). In the GG diet group, alkenylacyl-GPE and alkylacyl-GPE exhibited significantly lower ratios of SFA to PUFA compared to the controls (0.6 *vs* 1.3 for alkenylacyl-GPE, $P<0.01$, and 0.5 *vs* 1.2 for alkylacyl-GPE, $P<0.05$). There was also a decrease in the SFA/MUFA ratio (1.8 *vs* 2.5) in the alkylacyl group. Animals fed the SM diet exhibited a lower SFA/PUFA ratio in the both EPL subclasses compared to animals fed the control diet. There was no effect of SM or GG treatments observed on the ratio of SFA/MUFA or SFA/PUFA in the diacyl-GPE class.

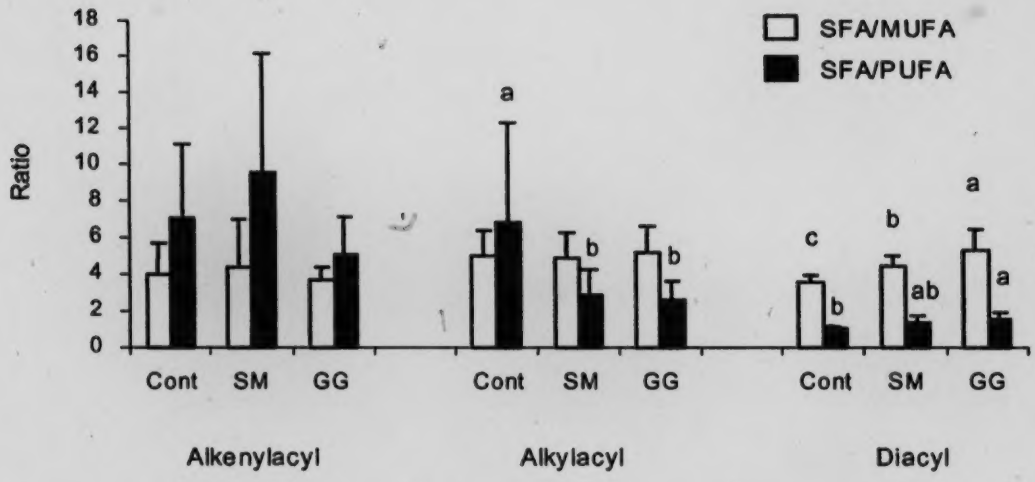

Figure VI-1. Ratio of SFA to MUFA (white columns) and SFA to PUFA (black columns) in alkenylacyl-, alkylacyl- and diacyl-GPC in intestinal mucosa of animals fed control or treatment diets. Values are means ± SD of 7, 8 and 7 animals for the control, SM and GG diet, respectively. Letters represent a significant difference between groups at $P<0.05$, except for the ratio of SFA to MUFA in the diacyl subclass at $P<0.001$.

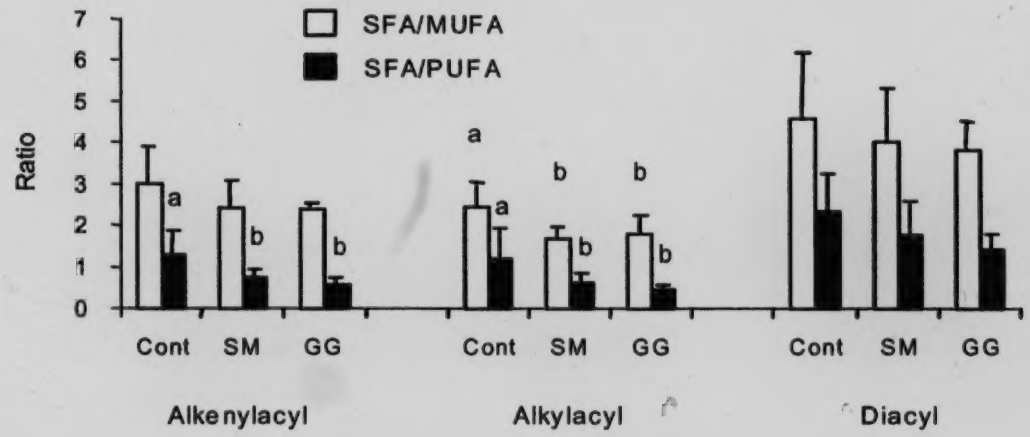

Figure VI-2. Ratio of SFA to MUFA (white columns) and SFA to PUFA (black columns) in alkenylacyl-, alkylacyl- and diacyl-GPE in intestinal mucosa of animals fed control or treatment diets. Values are means ± SD of 7, 8 and 7 animals for the control, SM and GG diet, respectively. Letters represent a significant difference between groups at $P<0.01$, except for the ratio of SFA to PUFA in the alkylacyl subclass at $P<0.05$.

6.4. DISCUSSION

The present study demonstrates that dietary SPL increases the biosynthesis of EPL in developing rat intestinal mucosa probably due to two possible mechanisms. First, it is assumed that hexadecanal, a derivative of SPL, is directly utilized for synthesis of EPL as a precursor of ether-linked fatty alcohols (7, 8). Unexpectedly, a ten times higher SPL dose in the SM diet than that of the GG diet was less effective at enhancing EPL content. This may suggest that there is another pathway to increase EPL synthesis. Secondly, it is possible that cholesterol reduction in intestinal cells caused by dietary SPL may increase EPL synthesis or uptake. Earlier studies show that a decrease in cholesterol content increases EPL uptake in human leukemia cell lines (28, 29). Since experiments in our laboratory have shown a cholesterol reduction in the intestine of animals fed the SPL diet (31), it is logical to assume that dietary SPL may increase EPL synthesis by decreasing intestinal cholesterol content. A recent study demonstrates that intestinal (I)-fatty acid binding protein (FABP) and liver (L)-FABP increase EPL content in PC and PE (40). It is suggested that dietary ganglioside may activate these two FABP expression in the intestine.

Elevated EPL level could alter inflammatory response. In this regard, alkylacylglycerol, an analogue of diacylglycerol (DG) which is derived from EPL by phospholipase C (PLC), is known to have a potent inhibitory effect on lipoxygenase (41) and cytosolic phospholipase A2 (cPLA2) activity (42), both of which stimulate an inflammatory response. Increased EPL content may enhance anti-oxidative effects on lipid peroxidation (25, 26) induced by many food-borne toxic or oxidative materials.

Higher levels of PUFA in EPL in animals fed the GG diet may have resulted from a decrease in total cholesterol content in intestinal mucosa which is known to cause a corresponding increase of Δ-5 and Δ-6 desaturase activities (30). Increased PUFA in EPL may be due to inhibition of PLA2 activity since ganglioside inhibits arachidonic acid-specific PLA2 activity (43) especially plasmalogen-selective PLA2 (44). The activity of plasmalogen-selective PLA2 is significantly inhibited by gangliosides or N-acetyl neuraminic acids derived from gangliosides (45, 46). In rat tissues, intestinal brush border has higher activity of calcium-independent cytosolic phospholipase A2 by 4~16 fold compared to stomach and spleen and about by 50~90 fold compared to lung, liver,

brain, kidney and heart (47). The present result showing increased 20:4n-6 in animals fed the GG diet may have resulted from attenuation of PLA2 activity (43, 44) or activation of I-FABP and L-FABP expression (40) in the intestine. I-FABP and L-FABP preferentially increase 20:4n-6 in PC and 20:4n-6 and 22:6n-3 in PE, respectively. In this regard, the present study suggests that intestinal mucosa from the weanling rats may have enough desaturase activity. The present finding that dietary gangliosides promoted a higher level of 20:4n-6, 22:4n-6 and 22:6n-3 in EPG raises the question of whether dietary ganglioside enhances FABP expression during gut development.

PUFA preferentially incorporated into alkenylacyl- and alkylacyl-PE compared to both classes of PC. Animals fed the GG diet showed an increase in total PUFA content in both classes of PE by 56% and 58%, respectively, compared to control animals accounted for 39% and 41%, respectively (Table VI-5, $P<0.01$). This result agrees with previous studies demonstrating more PUFA in EPL in PE than in PC (36, 48). The effect of dietary ganglioside on total PUFA content was different between diacyl-PC and diacyl-PE. Animals fed dietary ganglioside exhibited a decrease in total PUFA in diacyl-PC and an increase in diacyl-PE. This observation suggests that dietary ganglioside induces more incorporation of SFA into PC which is mostly localized at the outer membrane and of PUFA into PE situated at the inner membrane. Increase in total PUFA content resulted in a decrease in the ratio of total SFA/PUFA in EPL (Figure VI-1 and Figure VI-2).

The present study demonstrates that dietary ganglioside increases total and relative percentage of EPL and the PUFA content of EPL in intestinal mucosa during early development. These results suggest that dietary ganglioside influences gut development and protection by enhancing EPL content which has a preventative role in carcinogenesis (19, 22, 49), inflammation (41-43, 50) and lipid oxidation (25-27). The rate of metabolic conversion from ganglioside to EPL in the intestine is not known. Future investigations are needed to determine the conversion rate during age dependent gut development and if dietary ganglioside affects FABP expression in developing intestine.

6.5. REFERENCES

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CHAPTER VII

DIETARY GANGLIOSIDE DECREASES CHOLESTEROL CONTENT, CAVEOLIN EXPRESSION AND INFLAMMATORY MEDIATORS IN RAT INTESTINAL MICRODOMAINS

7.1. INTRODUCTION

Microdomains, generally called lipid rafts, caveolae, or glycosphingolipid-signaling domains, are important regions for signal transduction and lipid and protein trafficking (1-4). Microdomains have been recently recognized as one of the sites for cellular entry of bacterial and viral pathogens (5-7). For example, the entry of *filoviruses* occurs at sites of lipid rafts (7). Cholera toxin enters the cell by endocytosis and requires GM1 for retrograde trafficking into host cells via association with lipid rafts (8).

Physiological and functional roles of microdomains are dependent on cholesterol and sphingolipids, including gangliosides. Reduction of cholesterol inhibits pathogen entry by disrupting the structure of microdomains (9,10) and impairs inflammatory signalling (8,11). Cholesterol upregulates the expression of caveolin, a protein marker of caveolae (12,13). Sphingolipid depletion inhibits the intracellular trafficking of GPI-anchored proteins (14), suggesting that lipid-protein interaction directly modulates gene expression and cellular trafficking important for cell development and behaviour.

The neonatal intestine has endocytic and enzymatic transport systems for absorption of nutrients and immunoglobulins (15,16) but is susceptible to pathogen entry because of higher permeability than adult intestine (17). Gangliosides from mothers' milk may act as receptors for viral and bacterial toxins to protect against entry of pathogens into the neonatal enterocyte (18). During development, membrane permeability gradually decreases (17) while peptidases and glycosidases become functionally active and enriched in microdomains (19). Many digestive/absorptive enzymes, such as alkaline phosphatase, aminopeptidase N and A, and sucrase-isomaltase are also increased in apical membrane microdomains (20). A recent study demonstrates that the annexin-2 and caveolin-1 complex in microdomains regulates intestinal cholesterol transport (21). These results suggest the importance of microdomains in intestinal apical membranes for nutrient uptake and metabolism.

Long-chain polyunsaturated fatty acids (LCP, arachidonic acid and docosahexaenoic acid) can accumulate in microdomains and displace functional proteins by changing the lipid composition of the microdomain (22,23). This observation highlights the importance of dietary lipids in modulating physiological and biological properties of lipids and proteins in the microdomain. Whether dietary gangliosides affect the lipid profile and protein components of microdomains during gut development is not known.

Our previous study (Chapter III) showed that dietary ganglioside significantly increased total amount of gangliosides and decreased cholesterol content in the intestinal mucosa. Cholesterol depletion inhibits inflammatory signaling by disrupting microdomain structure (8,10,11). Thus, it was hypothesized that diet-induced cholesterol reduction in the microdomain would disrupt microdomain structure and reduce pro-inflammatory mediators such as diglyceride (DG) and platelet activating factor (PAF). DG derived from phospholipids by phospholipase C (PLC) binds to protein kinase C (PKC) to phosphorylate targeted proteins, such as the epidermal growth factor receptor and DG resides in microdomains (24,25). PAF, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphorylcholine, stimulates inflammatory cells such as leukocytes (26) and activates phospholipase A2 (PLA2) in the intestinal tissue to release arachidonic acid (27). Increased arachidonic acid and derivatives of the lipoxygenase pathway such as LTB4 and 5-HETE, simultaneously stimulate PAF synthesis by activation of acetyl-CoA acetyltransferase (28). PAF binds its receptor to increase intracellular calcium and inositol triphosphate (IP3) production and PKC activation for inflammation (29). It is unknown whether PAF also localizes in the microdomain. Since several studies reported that sphingomyelin (SM), a sphingolipid, has an inhibitory effect on PLA2 activity (30) and that PLA2 localizes in microdomains (31), it is of interest to determine whether dietary ganglioside also decreases PAF synthesis either by increasing sphingolipids or by disrupting microdomain structures in developing intestine. We also examine whether dietary ganglioside reduces DG content in the microdomain since sphingolipids inhibit PKC and PLC (32-34) and these two enzymes exist in microdomains (35).

Neonates consume sphingolipids, including gangliosides, from mothers milk (36,37). Gangliosides are known to act as receptors for viruses and toxins (38,39),

activators for T-cells (40) and stimulators for Th-1 and Th-2 cytokine-secreting lymphocytes in neonates (41). Gangliosides are also one of the major lipid components in microdomains. It is not known if dietary ganglioside changes the lipid profile and structure of the intestinal microdomain and modulates inflammatory signalling mechanisms in the developing intestine. The objective of the present study was to determine the effects of dietary ganglioside and LCP on microdomain structure and pro-inflammatory mediators, DG and PAF, in the developing gut.

7.2. MATERIALS AND METHODS

7.2.1. Animals and diets

This study was approved by the University of Alberta Animal Ethics Committee. Male Sprague-Dawley rats (18-day-old, n=8), with average body weights of 41.6 ± 1.6 g, were randomly separated into 3 groups of 8, with 2 or 3 rats housed in each polypropylene cage. Animals were maintained at a constant temperature of 23°C and a 12 h light/dark cycle. Animals had free access to water and one of three semi-purified diets containing 20% (w/w) fat for two weeks. The composition of the basal diets fed has been previously reported (42). Animal body weight and food intake were recorded every other day throughout the experiment. The control diet fat was a blend of triglyceride,

Table VII-1. Composition of experimental diets [1]

	Control	LCP	GG
Basal diet (g/100g)	80.0	80.0	80.0
Triglyceride	20.0 (100) [3]	20.0 (100)	19.9 (99.6)
20:4n-6	-	0.2 (1.0)	-
22:6n-3	-	0.1 (0.5)	-
Ganglioside	-	-	0.02 (0.1)
Phospholipid	-	-	0.05 (0.25)
Cholesterol	-	-	tr [2] (0.002)

[1] The composition of the basal diet has been previously published (43). [2] tr represents trace amount. [3] Values in parenthesis represent the percentage of total fat.

which reflected the fat composition of an existing infant formula. Fatty acids of the control diet (44) were composed of about 31% saturated fatty acids, 48% monosaturated fatty acids and 21% polyunsaturated fatty acids providing a ratio of 18:2n-6 to 18:3n-3 of 7 to 1. The long-chain polyunsaturated fatty acid diet (LCP diet) was formulated by adding 1% (w/w) arachidonic acid (20:4n-6) and 0.5% (w/w) docosahexaenoic acid (22:6n-3) (Martek Biosciences, USA) to a control diet. The ganglioside diet (GG diet) was formulated by adding ganglioside (New Zealand Dairy, New Zealand) to the control diet at a level of 0.1% (w/w of total fat) ganglioside in the total diet. In the GG diet, the phospholipid and cholesterol content was 0.25% and <0.002% w/w of total fat, respectively. The ganglioside fraction contained approximately 80% (w/w) GD3. GD1b, GM3, and other minor gangliosides (GM2, GM1 and GT1b) constituted 9%, 5% and 6% (w/w), respectively.

7.2.2. Collection of samples

After anesthetising animals with halothane, the small intestine (jejunum to ileum) was excised. The intestine was washed with ice cold 0.9% saline solution to remove visible mucus and dietary debris, opened and moisture was carefully removed with a paper towel to measure mucosa weight. Intestinal mucosa was scraped off with a glass slide on an ice cold glass plate. All mucosa samples were kept at −70°C until extraction.

7.2.3. Sucrose gradient separation of microdomains

Intestinal microdomains were prepared by ultra-centrifugation of a discontinuous sucrose gradient (45). Intestinal mucosa was suspended with TME (10 mM Tris-HCl, 1 mM $MgCl_2$, 1 mM EGTA) solution containing 1 mM phenylmethyl sulfonyl fluoride, 0.001% (w/v) apotinin and 2% (v/v) Triton X-100 for 30 min in ice and homogenized with 15 strokes of a Dounce homogeniser with a tight-fitting pestle (Wheaton Scientific, USA). The homogenate was adjusted to 45% (w/v) sucrose by adding an equal volume of 90% (w/v) sucrose and then homogenized again with 5 strokes of the Dounce homogeniser. A 5-35% discontinuous sucrose gradient was overlaid on the homogenate in 45% (w/v) sucrose, which left a 45-35-5% sucrose gradient from the bottom. After 16h centrifugation at 70,000 × g at 4°C in a Beckman SW 28 Ti rotor, the interface between 5

and 35% sucrose was collected as the microdomain fraction. Microdomains were washed with TME solution and centrifuged twice at 100,000 × *g* for 1 h at 4°C to remove sucrose and Triton X-100. The pellet was resuspended in phosphate buffer solution and used for protein and lipid analysis. Enrichment of microdomains was confirmed by analyzing the amounts of caveolin, cholesterol and gangliosides in the intestinal microdomain compared to the protein pellet which was soluble in detergent solution. In immunoblotting and TLC development to confirm the method for microdomain purification, the content of caveolin-1 and cholesterol was 4~5 times higher in the intestinal microdomain fraction than the detergent soluble protein pellets (Figure VII-1-A and C). Intestinal gangliosides were exclusively found in the microdomain and not in the detergent soluble protein pellet by a densitometry assay on TLC plates (Figure VII-1-B).

7.2.4. Western blotting for caveolin content

Protein content from microdomains was measured by QuantiPro BCA Assay Kit (Sigma-Aldrich Co., MO, USA). Twenty or thirty μg protein was dissolved with SDS reducing sample buffer and loaded onto 15% SDS-PAGE minigels. After transferring proteins onto nitrocellulose (Amersham Pharmarcia Biotech, UK), membranes were blocked with 5% non-fat dried milk in TBS-T (20 mM Tris; pH 7.6; 137 mM NaCl; 0.1% Tween 20) for 1 h at room temperature. The primary antibody (BD Bioscience, ON, Canada), which specifically recognizes caveolin, was diluted 1:1000 in TBS-T with 1% non-fat dried milk and incubated for 90 min at room temperature. The membrane was washed three times for 10 min each time in TBS-T. The secondary antibody (goat anti-mouse IgG-HRP conjugate; Bio-Rad, CA, USA) was diluted 1:2000 in 1% non-fat dried milk in TBS-T and incubated for 1 h at room temperature. After washing the membrane with TBS-T three times for 10 min each time, the caveolin protein was developed by enhanced chemiluminescence (ECL) detection reagent according to the protocol supplied by Amersham Pharmarcia Biotech, UK. Blot intensity of caveolin was measured from five animals from each diet group using an Imaging Densitometer (Bio-Rad, CA, USA).

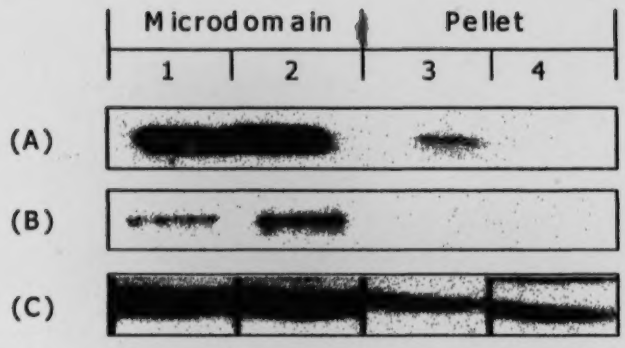

Figure VII-1. Comparison of total content of caveolin-1 (A), ganglioside (B) and cholesterol (C) in the microdomain fraction (lane 1 and 2) and the pellets precipitated (lane 3 and 4) from intestinal mucosa. The image was obtained by immunoblotting for caveolin-1 and by TLC development for ganglioside GM3 and cholesterol analysis. Ganglioside GM3 and cholesterol bands on the TLC plate were visualized by spraying resorcinol-HCl and 10% $CuSO_4$5H2O in 8% H_3PO_4 solution, respectively. TLC solvent system was chloroform/methanol/0.2% (w/v) $CaCl_2 \cdot 2H_2O$ (55:45:10, by vol.) for ganglioside and chloroform: methanol:H_2O (60:35;8, by vol.) and chloroform/methanol/ acetic acid (90:2:8, by vol.) for cholesterol.

7.2.5. Ganglioside extraction and purification

Total lipid was extracted from the microdomain fraction using the Folch method (46). For extraction of gangliosides (47), the upper phase was collected into a test tube and the lower organic phase was washed twice with the Folch upper phase solution (chloroform/methanol/water, 3:48:47 by vol.). The upper phase gangliosides were pooled and purified by passage through Sep-Pak C_{18} cartridges (Waters Corporation, Milford, MA, USA) preconditioned with 10 mL of methanol, 20 mL of chloroform/methanol (2:1, v/v), and 10 mL of methanol as described by Williams and McCluer (48). Cartridges were washed with 20 mL of distilled water. Gangliosides were eluted with 5 mL of methanol and 20 mL of chloroform/methanol (2:1, v/v), dried under N_2 gas and then redissolved in 500 µL of chloroform/methanol (2:1, v/v). Gangliosides were stored at -70°C until analysis.

7.2.6. Analysis of total and individual ganglioside content

Total ganglioside content was measured as ganglioside bound N-acetyl neuraminic acid (NANA) as described by Suzuki (49). An aliquot of purified ganglioside sample was dried under N_2 gas and dissolved with 0.5 mL distilled H_2O and 0.5 mL resorcinol-HCl (50) in screw-capped Teflon-lined tubes. The purple-blue color developed by heating was extracted into butylacetate/butanol (85:15, v/v). Optical density was read at 580 nm. Individual gangliosides were identified by silica gel high performance thin layer chromatography (HPTLC; Whatman Inc, Clifton, NJ, USA) using ganglioside standards GM3, GM2, and GD3 and bovine brain ganglioside mixture (Alexis, San Diego, CA, USA) in a solvent system of chloroform/methanol/0.2 % (w/v) $CaCl_2 \cdot 2H_2O$ (55:45:10, by vol.). Individual gangliosides were recovered and measured as described above.

7.2.7. Cholesterol assay

Cholesterol analysis was completed with a test kit (Sigma, MO, USA).

7.2.8. Analysis of sphingomyelin (SM) and platelet activating factor (PAF)

To determine sphingomyelin content, total lipid extracted from microdomains was applied onto a silica gel 'H' TLC plate and separated using chloroform/methanol/2-propanol/0.2% KOH/triethylamine (45:13.5:37.5:9:27, by vol.). PAF was separated from the total lipid fraction on a silica gel 'G' TLC plate (Fisher Scientific, CA) using chloroform/methanol/water, (65:35:6, by vol.) for PAF. Commercial standards of SM, PAF, and lyso-PC (Sigma, MO, USA) were spotted onto the plate for identification. TLC plates were visualized with 0.1% ANSA (anilino naphthalene sulfonic acid) under UV exposure. Lipids were recovered and lipid phosphate was measured according to the method of Itoh et al. (51).

7.2.9. Analysis of diglyceride (DG)

To measure DG content, total lipid was separated on a silica gel 'G' TLC plate using petroleum ether/diethyl ether/acetic acid (80:20:1, by vol.). 1,2-DG and 1,3-DG were exposed to 0.1% ANSA and identified under UV light by comparison with commercial standards. Cholesterol comigrated with 1,3-DG, so these were recovered in a

single fraction. 1,2-DG and 1,3-DG were methylated with a known amount of heptadecanoic acid (C17:0) as an internal standard. To remove cholesterol from 1,3-DG after methylation, fatty acid methyl esters (FAME) were applied onto a silica gel 'G' plate and developed with toluene. The purified FAME fraction was collected, extracted with hexane and injected into a gas liquid chromatograph (GLC, Varian Model 3400 CX, CA) to measure total fatty acid content in DG. The GLC was equipped with a flame ionization detector and a 25 m BP-20 fused capillary column (SGE, Australia).

7.2.10. Statistical analysis

Values shown are means ± standard deviation (SD). Significant difference between the control group and experimental groups was determined by one-way analysis of variance (ANOVA) with SAS (SAS Institute Inc, Version 8.2, NC, USA). Significant effects of diet treatment were determined by a Duncan multiple range test at a significance level of $P<0.05$.

7.3. RESULTS

7.3.1. Animal growth and tissues

The initial and final body weights of animals or food consumption after two weeks feeding of experimental diets was not significantly different among control, GG, and LCP groups. Intestinal mucosal weight and intestinal length were not affected by dietary treatment (data not shown).

7.3.2. Ganglioside content and composition

Animals fed the GG diet had a 50% increase in total ganglioside content in intestinal microdomains (Figure VII-2). Relative content of GM3 in microdomains decreased significantly from 83.7% to 77.7%, while GD3 increased from 4.4% to 8.3% (Table VII-2). These changes in ganglioside profile were accompanied by a significant increase in GD3 content (Figure VII-4-A), but GM3 content remained the same (data not shown). This finding agrees with the previous study (Chapter III), in which it was found that dietary ganglioside significantly increased total ganglioside content in developing rat intestinal mucosa resulting

in compositional changes such as decreased GM3 and increased GD3. The content of GM1, GD1a, GD1b and GT1b was not changed by either experimental diet (Table VII-2). The LCP diet had no effect on total ganglioside content or relative composition.

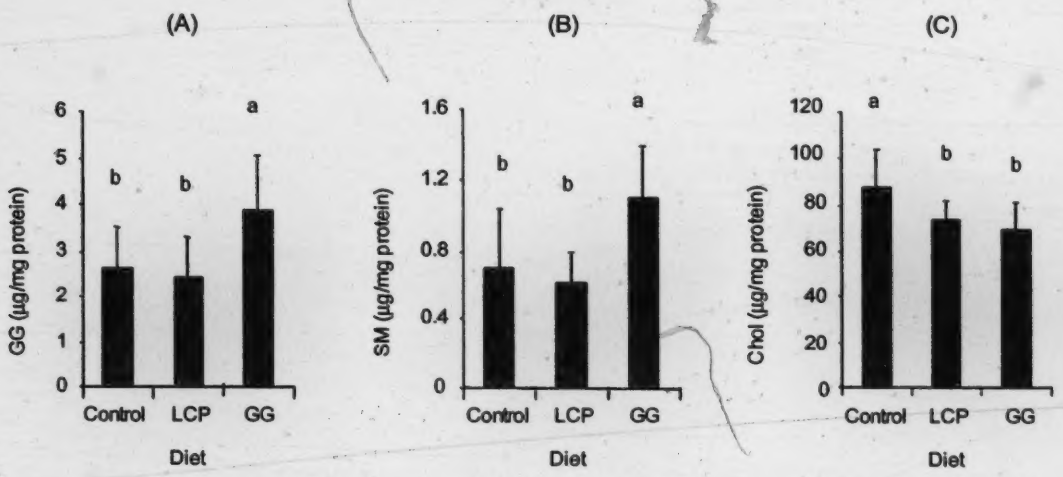

Figure VII-2. Total content of (A) ganglioside, (B) sphingomyelin and (C) cholesterol in intestinal microdomains after two week diet treatment. Values are presented as means ± SD where n=8 animals in each group. Differences are significant at levels $p<0.01$, $P<0.01$, and $P<0.05$ for (A), (B), and (C), respectively.

Table VII-2. Composition of gangliosides in rat intestinal microdomains after diet treatment

Ganglioside (%)[1]	Control	LCP	GG	Diet effect (p)
GM3[2]	83.7 ± 2.7[a]	80.3 ± 4.0[ab]	77.7 ± 3.3[b]	0.05
GM1	2.7 ± 0.7	2.1 ± 1.9	2.7 ± 1.4	
GD3	4.4 ± 1.0[b]	4.5 ± 1.4[b]	8.3 ± 2.1[a]	0.01
GD1a	3.5 ± 1.0	3.6 ± 1.0	3.5 ± 1.2	
GD1b	3.5 ± 1.1	5.6 ± 2.1	4.2 ± 1.9	
GT1b	2.2 ± 1.9	3.9 ± 2.1	3.5 ± 0.5	

[1] Values are expressed as a % of total NANA in ganglioside fraction and represent means ± SD of 8 rats. Within a row, values with different superscript letters are significantly different. [2] Nomenclature is described by Svennerholm (47).

7.3.3. Sphingomyelin content

Animals fed the GG diet showed a 57% increase in sphingomyelin content in microdomains (Figure VII-2), whereas the LCP diet had no effect on sphingomyelin.

7.3.4. Cholesterol content of microdomains

Both the GG diet and the LCP diet resulted in a significant lowering of microdomain cholesterol levels, but the effect of the LCP diet was slightly less pronounced (Figure VII-2).

7.3.5. Cholesterol/ganglioside ratio and cholesterol/sphingomyelin ratio

Animals fed the GG diet showed a 40% reduction in the ratio of cholesterol to total gangliosides, from 31.3 to 18.9 (Figure VII-3), whereas the LCP diet had no effect on this relationship. Feeding the GG diet also resulted in a 56% reduction in the ratio of cholesterol to sphingomyelin. The ratios were 143, 117 and 63 for control, LCP, and GG diets, respectively (Figure VII-3).

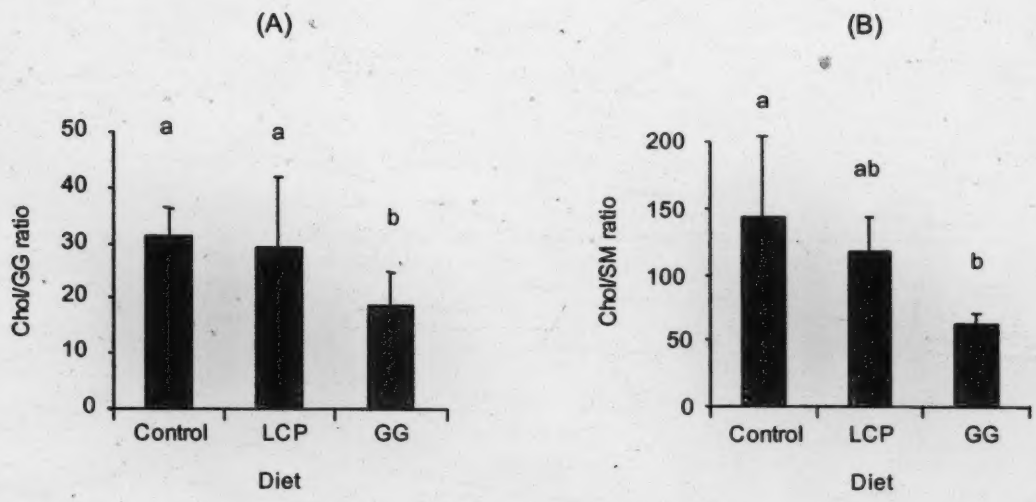

Figure VII-3. The ratio of (A) cholesterol/ganglioside and (B) cholesterol/sphingomyelin in intestinal microdomains after two week diet treatment. Values are presented as means ± SD where n=8 animals in each group. Differences are significant at levels $p<0.05$ and $p<0.01$ for (A) and (B), respectively.

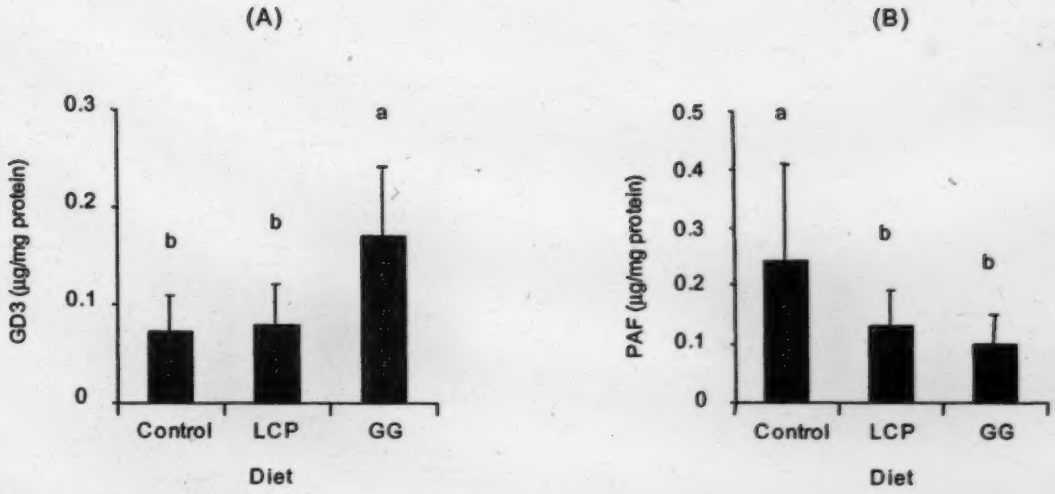

Figure VII-4. Total content of (A) GD3 and (B) PAF in intestinal microdomains after two week diet treatment. Values are presented as means ± SD where n=8 animals in each group. Differences are significant at levels $P<0.01$ and $P<0.05$ for (A) and (B), respectively.

7.3.6. Content of pro-inflammatory mediators, diglyceride (DG) and platelet activating factor (PAF)

After feeding the GG diet, total DG and 1,2-DG were reduced by 43% and 44%, respectively (Table VII-3). The LCP diet also caused a reduction in these two lipids but to a lesser extent (32% and 33% for total DG and 1,2-DG, respectively). The GG and LCP diets decreased PAF content by 59% and 47%, respectively (Figure VII-4).

Table VII-3. Content of diglycerides (µg/mg protein) in rat intestinal microdomains after diet treatment [1]

	Control	LCP	GG
1,2-diglyceride	15.4 ± 2.3^a	10.4 ± 3.3^b	8.6 ± 4.4^b
1,3-diglyceride	1.7 ± 0.2	1.3 ± 0.6	1.2 ± 0.2
total diglyceride	17.1 ± 2.2^a	11.7 ± 3.8^b	9.8 ± 4.6^b

[1] Values are means ± SD of 8 rats. Within a row, values with different superscript letters are significantly different at $P<0.01$.

7.3.7. Caveolin content in microdomains

Both experimental diets were associated with significantly lower expression of caveolin protein (70% and 60% reduction for the LCP diet and GG diet, respectively) in intestinal microdomains (Figure VII-5).

7.4. DISCUSSION

Diet–induced change in the microdomain ganglioside content is significant because GD3 and GM3 are involved in a variety of cellular functions. GM3 is colocalized with signalling molecules such as c-Src, Rho, and Fak in microdomains (52,53). Thus, reduction in GM3 by the GG diet may alter signalling pathways related to these molecules. The elevation of GD3 by the GG diet may enhance immune function and gut protection during development since GD3 activates T-cells (40) and has an anticarcinogenic effect in the mouse colon (54). Our results showing accumulation of dietary gangliosides in microdomains are supported by a previous study demonstrating

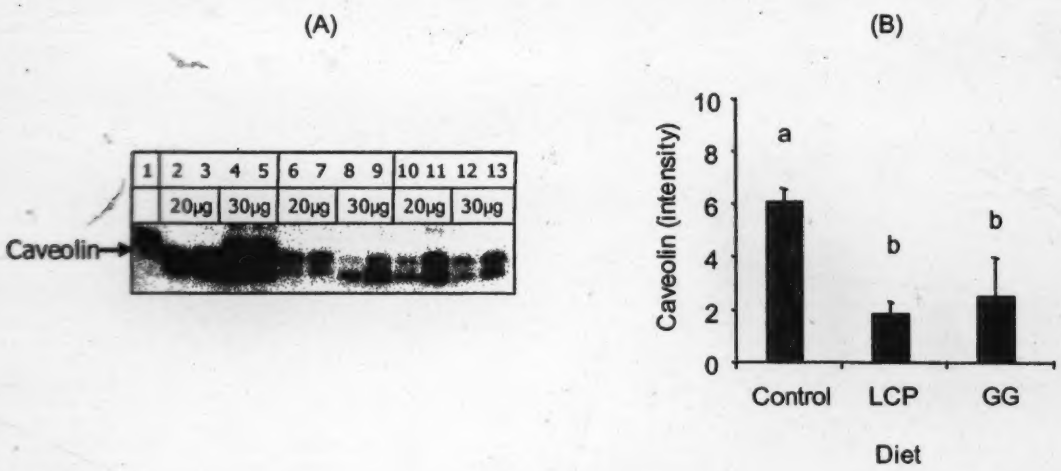

Figure VII-5. Western blot of caveolin content in intestinal microdomains after diet treatment for two weeks. Lane 1, caveolin standard (21-24 kDa); lane 2-5, control diet; lane 6-9, LCP diet; lane 10-13, GG diet. Two different sample amounts (20 and 30 μg) were subjected to Western blot analysis from each group. Relative change in caveolin content was determined by image densitometry. Values are presented as means ± SD for five animals (n=5) in each treatment group ($P<0.01$).

that administration of [3H]GM3 to Neuro 2a cells showed enrichment of [3H]GM3 in microdomains (55). These observations suggest that an exogenous supplement of ganglioside is directly incorporated into the microdomain.

Cholesterol is an important lipid involved in compartmentalizing lipids and proteins into microdomains (56). Recent studies found that viral pathogens and cholera toxin appear to reach the ER by caveolae (57,58). Cholesterol reduction in cell membranes inhibits the invasion of HIV-1 (9), cholera toxin (8), and malarial parasite (10) by disrupting microdomain structure. Cholesterol depletion by drugs has been shown to down-regulate caveolin gene expression (13). A similar effect is reported here in that cholesterol depletion by diet modification created a reduction in caveolin protein expression. Decreased caveolin expression may induce a dissociation of the complex of annexin-2 and caveolin-1 necessary for cholesterol trafficking in the intestine (21). Thus, by reducing microdomain cholesterol content leading to lower caveolin expression and disruption of potential invasion sites, a possible anti-infective effect of dietary ganglioside is demonstrated.

Our study provides new evidence to indicate that dietary ganglioside and LCP decrease pro-inflammatory mediators PAF and DG in the intestinal microdomain of developing animals. The ganglioside-enriched diet is more effective at reducing these factors than the LCP-enriched diet. It is known that GM3 stimulates PLA2 activity, leading to arachidonic acid release in human peripheral blood lymphocytes (59). Thus, the decrease in GM3 observed in the microdomain may be linked to reduction in PLA2 activity which in turn leads to decreased PAF content (Table VII-3). Since it is known that PAF binds to a PAF receptor in cell membranes to initiate inflammatory signalling events (29), this study suggests that PAF receptors localize in the intestinal microdomain. Recent studies have demonstrated that GD3 has a stronger inhibitory effect against PKC activation than GM3 (34) and that gangliosides in general appear to be inhibitory to PLC activity (32). The increase in total gangliosides and GD3 observed in association with the GG diet may be related to the reduction in DG content through inhibition of PKC or PLC. Microdomain DG might be a critical lipid component for modulating the structure and function of microdomains through activation of PKC (33), since PKC regulates a cyclic

transition of microdomains from the membrane to a vesicle that then returns to the cell surface (25).

The potential for dietary supplementation of physiologic doses of dietary ganglioside to exert anti-inflammatory effects in the gut of developing animals has been described. These results suggest that dietary ganglioside is bioavailable in the gut and has potential protective roles in the neonatal intestine.

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CHAPTER VIII

DIETARY GANGLIOSIDE INHIBITS PRO-INFLAMMATORY SIGNALS, PAF, PGE2, LTB4, IL1-β AND TNF-α IN THE INFLAMED INTESTINE AND BLOOD INDUCED BY LPS TOXIN EXPOSURE AFTER 6 H

8.1. INTRODUCTION

Gangliosides are widely distributed at the surface of mammalian cells and play roles in modulating cell adhesion, signal transduction and receptors for bacterial and viral toxins (1-3). The content and composition of gangliosides vary in species, tissues, and with age (4-6). The content of ganglioside increases during neonatal intestine development (7). The ganglioside content in rat intestinal mucosa reaches a maximum level at about 7 days postnatal age and declines from this maximum 4 to 5 fold by 30 days postnatal age (7). During early lactation human milk contains about 2~3 fold higher content of ganglioside compared to mature milk (8). The early appearance of ganglioside in neonatal tissues and milk may suggest important roles for gangliosides in growth and protection of neonates. Early appearance of gangliosides from birth to about 2 weeks of age overlaps with the time when neonates do not have immunoglobulin-producing cells (9). This suggests that gangliosides may influence immune function and development. Earlier studies show that infants fed ganglioside-supplemented formula had significantly lower content of fecal *Escherichia coli* (*E.coli*) than infants fed control milk formula at 7 days after birth (10). Fecal *bifidobacteria* counts were higher in infants fed ganglioside-supplemented formula at age 30 days of postnatal age (10). Gangliosides in milk may play a role in protecting neonatal gut development from antigens and toxins (10,11). No studies have been done to determine if dietary ganglioside protects gut from endotoxin during early development.

Several in vitro studies suggesting anti-inflammatory effects of gangliosides (12-15). When human epithelial cells are incubated with buffalo milk ganglioside compared to controls, PGE2 production is inhibited by 75% in response to TNF-α (12). Ganglioside inhibits plasmalogen-selective PLA2 from bovine brain in vitro (15) and arachidonic acid-specific PLA2 in cultured neuroblastoma cells (16). In Fukushima study (14), the small intestine appears to possess a calcium-independent cPLA2 specific for AA release

from ether phospholipids. The calcium-independent cPLA2 activity of the intestine is much higher than 12 other tissues tested including stomach, spleen, lung, brain and muscles by 4~430 times (14). No studies have been done to examine if dietary ganglioside has anti-inflammatory effects on platelet activating factor (PAF), PGE2, LTB4, IL-1β, and TNF-α in gut and blood of animals treated with LPS toxin during early development.

A previous experiment (Chapter VII) shows that dietary ganglioside increases total ganglioside content and decreases cholesterol, PAF content and caveolin-1 expression in intestinal microdomains (17). Cholesterol reduction or caveolae disruption inhibits entry of pathogens (18-21) and inflammatory signals into epithelial cells (22,23). PAF is a potent inflammatory lipid mediator which stimulates inflammatory signals during chronic, acute or endotoxin-induced inflammation (24-27). Thus, we hypothesized that dietary ganglioside-induced decrease in PAF, cholesterol content and caveolin-1 expression in microdomains will inhibit pro-inflammatory signals induced by LPS treatment in developing rat intestine. Reduction in pro-inflammatory signals released into the blood should also be observed.

8.2. MATERIALS AND METHODS

8.2.1. Animals and diets

This study was approved by the University of Alberta Animal Ethics Committee. Male Sprague- Dawley rats (18-day-old, n=16/diet group) were randomly separated into 2 groups of 16 with 2 or 3 rats housed in each polypropylene cage. Animals were fed a semi-purified diet containing 20% (w/w) fat for 2 weeks with ganglioside (GG diet) or without (control diet) ganglioside supplementation. The semi-purified diet fat was a blend of triglyceride, which reflected the fat composition of an existing infant formula. The composition of the basal diets fed has been previously reported (28). The treatment diet was formulated by adding ganglioside (GG diet; 0.1% w/w of total lipid, New Zealand Dairy, New Zealand) to the control diet. In the GG diet, the ganglioside GD3 fraction contained about 80% w/w of total gangliosides. GD1b, GM3 and other gangliosides (GM2, GM1 and GT1b) was 9, 5 and 6% w/w, respectively. The cholesterol content was negligible (<0.002% w/w of total lipid). After 2 weeks of feeding diets, half of the

animals from each group were randomly separated into two groups: a control group which received 0.9% saline solution by IP injection and a LPS group which were injected intraperitoneally with lipopolysaccharide (LPS) from *E.coli* serotype O111:B4 at 3 mg of LPS per Kg body wt. Animals had access to diets and water for 6 h after the injection. Over all, the LPS dosage given to animals was not lethal. However, for the first 24 h post LPS injection, animals became progressively lethargic. After 24 h, the animals gradually recovered until they behaved normally at 72 h. In a pilot experiment (n=3), upon surgical removal and inspection of the intestine, intestinal inflammation was minor at 6 h, severe at 24 h and disappeared at 72 h after LPS injection.

8.2.2. Sample collection

Six hour after LPS injection, animals were anesthetized with halothane, and blood was collected by cardiac puncture and immediately centrifuged at 1000 x g (JA 20 Rotor, Beckman, USA) for 30 min to recover plasma. Following decapitation, small intestines were excised. The intestine was washed with ice-cold 0.9% saline solution to remove visible mucus and dietary debris, opened longitudinally, and moisture was carefully removed with a paper towel to measure mucosa weight. Intestinal mucosa was scraped off with a glass slide on an ice-cold glass plate. All mucosa samples were weighed and kept in a –70°C freezer until extraction.

8.2.3. Ganglioside extraction and purification

Gangliosides were separated by the Folch method (29) and purified by passing through Sep-Pak C_{18} cartridges (Waters Corporation, Milford, MA, USA) prewashed with 10 mL of methanol, 20 mL of chloroform/methanol (2:1, v/v), and 10 mL of methanol as described by Williams and McCluer (30). Brifely, the upper phase extract was loaded onto C_{18} cartridges prewashed. Cartridges were then washed with 20 mL of distilled water to remove salts and water-soluble contaminants. Gangliosides were eluted with 5 mL of methanol and 20 mL of chloroform/methanol (2:1, v/v), dried under N_2 gas and then redissolved with 500 µL of chloroform/methanol (2:1, v/v). Gangliosides were stored at -70°C until analysis.

8.2.4. Analysis of total ganglioside content by measuring NANA

Total ganglioside bound NANA (GG-NANA) were measured as described by Suzuki (31). Ganglioside purified by Sep-Pak C_{18} cartridges was dried under N_2 gas and dissolved with each of 0.5 mL of distilled H_2O and resorcinol-HCl (32) in screw-capped Teflon-lined tubes. The purple blue color developed by heating was extracted into butylacetate/butanol (85:15, v/v) solvent. Optical density was read by a spectrophotometer (Hewlett Packard, 8452A) at 580 nm. For quantitative analysis, N-acetyl neuraminic acid (Sigma, MO, USA) was used as a standard.

8.2.5. Assay of PAF (platelet activating factor) content

PAF was separated from total lipid extracted from the mucosa using a silica gel 'G' TLC plate (Fisher Scientific, CA) using chloroform/methanol/water, (65:35:6, by vol.). A commercial standard of PAF (Sigma, MO, USA) was also spotted on the plate for identification. After TLC development, PAF was identified by 0.1% ANSA (anilino naphthalene sulfonic acid) spray and recovered for measuring phosphate content in PAF as described earlier (33). Optical density was read in a spectrophotometer (Hewlett Packard, 8452A) at 790 nm.

8.2.6. Extraction and assay of eicosanoids (PGE2 and LTB4)

Eicosanoids were extracted as previously described by Nieto (34). Tissues were homogenized in 10 volume of PBS (10 mM, pH 7.4, 0.001% indomethacin) with a Polytron homogenizer for 30 sec at 4°C. The 500 µL homogenate was dissolved with 4 volume of water/ethanol mixture (1:4, v/v) and 10 µL of glacial acetic acid was added. Samples were gently mixed, incubated at room temperature for 5 min and then centrifuged at 5000 rpm for 5 min at 4°C. The supernatant was applied onto Sep-Pak C_{18} cartridges prewashed with 10 mL H_20, 10 mL 15% ethanol and 10 mL hexane. Eicosanoids eluted with 10 mL ethyl acetate were dried and resolved in ethanol and assay buffer supplied by commercial ELISA kit sets (R&D Systems, MN, USA). Eicosanoids in blood plasma were applied directly to the ELISA test using a recommended dilution. Optical density was measured by a microplate reader (Molecular Devices Co., USA) at 405 nm for both PGE2 and LTB4.

8.2.7. Assay of cytokines (IL-1β and TNF-α)

Inflammatory cytokines, IL-1β and TNF-α in blood plasma were measured by following instructions supported by an ELISA kit from R&D Systems (MN, USA) and BD Biosciences (ON, CA) respectively. Optical density was measured by a microplate reader (Molecular Devices Co., USA) at 450 nm for both IL-1β and TNF-α.

8.2.8. Statistical analysis

The values shown are means ± standard deviation (SD). Significant differences between the control group and experimental groups were determined by a student t-test or nonparametric statistical analysis. Significant effects of diet treatment were determined at a significance level of $P<0.05$.

8.3. RESULTS

8.3.1. Ganglioside and PAF content

The effect of dietary ganglioside on total ganglioside content of intestinal mucosa inflamed by LPS from animals fed either the control or GG diet for 2 weeks is shown (Figure VIII-1-(A)). Animals fed dietary ganglioside showed a significant increase in total ganglioside content in intestinal mucosa by up to 40% ($P<0.05$) in the saline group and 89% ($P<0.001$) in the LPS group compared to animals fed the control diet. Feeding animals the GG diet significantly decreased total PAF content in the intestine inflamed by LPS both in the saline group by 37% ($P<0.0001$) and in the LPS group by 45% ($P<0.0001$) compared to control animals (Figure-VIII-1-(B)).

8.3.2. PGE2 and LTB4 content in intestinal mucosa and blood plasma

After 6 h LPS exposure, animals fed dietary ganglioside decreased PGE2 content in intestinal mucosa inflamed by LPS both in the saline animals (73 *vs* 64 pg/mg protein) and the LPS animals (84 *vs* 74 pg/mg protein) compared to animals injected with saline solution (Figure VIII-2-(A)). Animals fed the GG diet exhibited a significant reduction in LTB4 production after 6 h LPS injection in the saline group and the LPS group by 41% and 20%, respectively, compared to animals fed the control diet (Figure VIII-2-(B)).

In blood plasma, there was a decrease in PGE2 in the saline animals (75%) but not in the LPS animals fed the GG diet compared to animals fed the control diet (Figure VIII-3-(A)). Animals fed the GG diet exhibited a 41% inhibition of LTB4 6 h after LPS injection compared to animals fed the diet without gangliosides (Figure VIII-3-(B)).

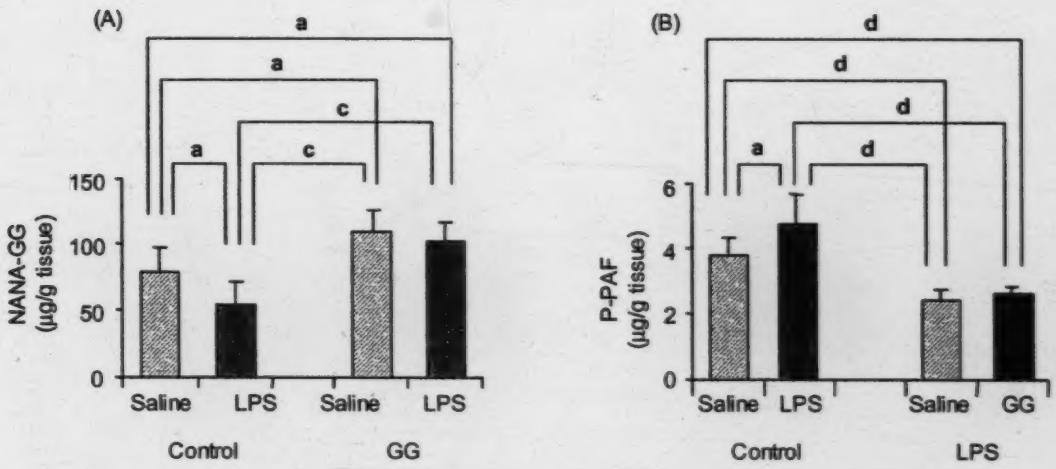

Figure VIII-1. The content of gangliosides (A) and PAF (B) in intestinal mucosa from animals after 6 h LPS injection. Gangliosides purified by Sep-Pak C_{18} cartridge were measured as NANA bound gangliosides described by Suzuki (31). PAF was separated on silica gel 'G' TLC plate using a solvent system, chloroform/methanol/water, (65:35:6, by vol.). After 0.1% ANSA (anilino naphthalene sulfonic acid) spray, PAF was identified by a commercial standard and recovered for measuring phosphate content in PAF as described (33). Optical density was read in a spectrophotometer at 580 nm and 790 nm, respectively. Statistical analysis was a two tailed Student t-test (a, $P<0.05$; b, $P<0.01$; c, $P<0.001$; d, $P<0.0001$).

8.3.3. IL-1β and TNF-α content in blood plasma

In animals fed the control diet, both IL-1β and TNF-α were increased in blood plasma in animals infected by LPS toxin for 6 h compared to animals injected with saline solution (Figure VIII-4-(A) and (B)). Animals fed dietary ganglioside significantly blocked IL-1β and TNF-α secretion after LPS exposure by 58% (322 *vs* 136 pg/mL, $P<0.01$) and 55% (148 *vs* 66 pg/mL, $P<0.05$) compared to animals fed the control diet.

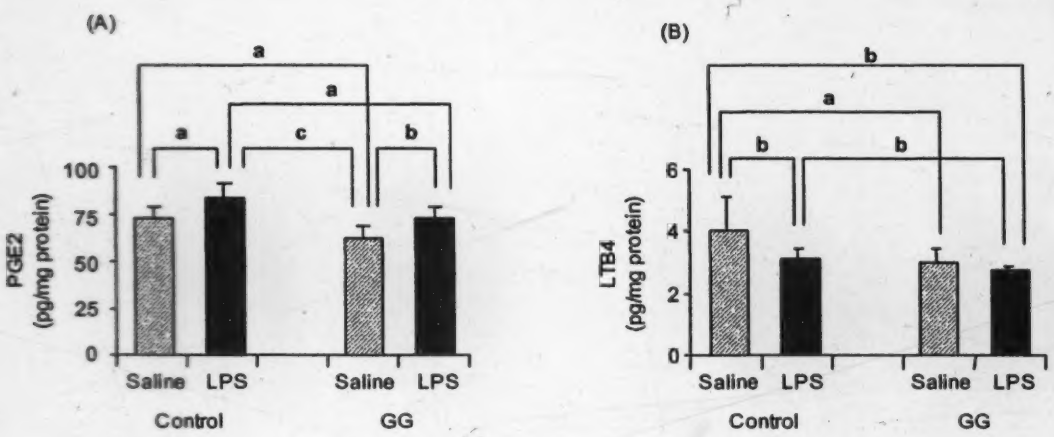

Figure VIII-2. The content of PGE2 (A) and LTB4 (B) in intestinal mucosa from animals after 6 h LPS injection. Mucosa homogenates in water/ethanol mixture (1:4, v/v) with 10 µL of glacial acetic acid (34) was incubated at room temperature for 5 min and then centrifuged at 5000 rpm for 5 min at 4 °C. The supernatant was applied onto Sep-Pak C18 cartridges prewashed with 10 mL H20, 10 mL 15% ethanol and 10 mL hexane. Eicosanoids eluted with 10 mL ethyl acetate were dried and resolved in ethanol and assay buffer supplied by commercial ELISA kit sets. Optical density was measured using a microplate reader at 405 nm for both PGE2 and LTB4. Statistical analysis was a two tailed Student t-test (a, $P<0.05$; b, $P<0.01$; c, $P<0.001$).

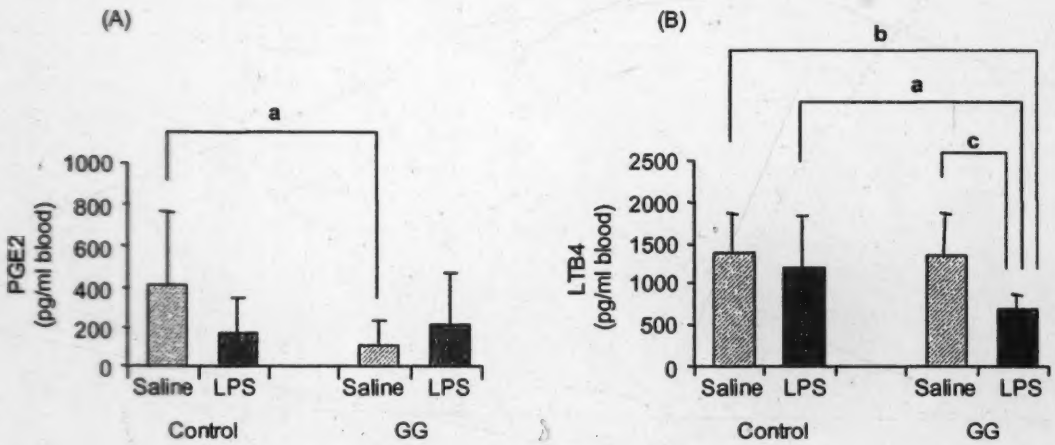

Figure VIII-3. The content of PGE2 (A) and LTB4 (B) in blood plasma from animals after 6 h LPS injection. Eicosanoids in blood plasma were directly applied to ELISA kit using recommended dilution. Optical density was measured using a microplate reader at 405 nm for both PGE2 and LTB4. Statistical analysis was a two tailed Student t-test (a, $P<0.05$; b, $P<0.01$; c, $P<0.001$).

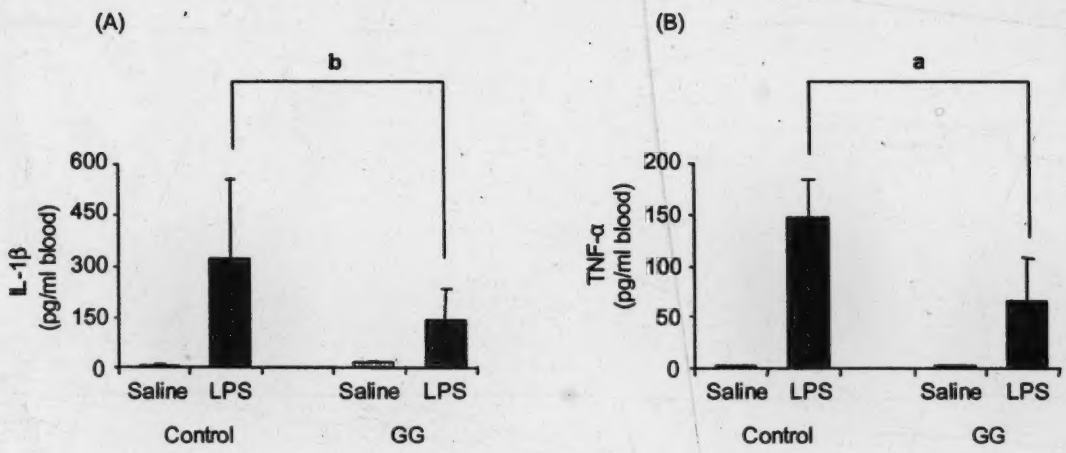

Figure VIII-4. The content of IL-1β (A) and TNF-α (B) in blood plasma from animals after 6 h LPS injection. IL-1β and TNF-α in blood plasma were directly applied to ELISA kit by using recommended dilution. Optical density was measured by a microplate reader at 450 nm for both IL-1β and TNF-α. Statistical analysis was a two tailed nonparametric analysis (a, $P<0.05$; b, $P<0.01$).

8.4. DISCUSSION

Animals exposed to enterotoxins from *Salmonella enteritidis, Salmonella typhosa* or *E.coli* produce a significant increase in PAF content in rat gastrointestinal tract, in turn leading to mortality and gastrointestinal damage (35,36). Administration of antagonists of PAF into animals reduces the damage (35,36). The present study found a significant reduction in PAF content by up to 45% ($P<0.0001$, Figure VIII-1-(B)) in animals fed dietary ganglioside compared to animals fed the control diet. The significant reduction in PAF content was probably due to an inhibition of PLA2 by increased ganglioside content of intestinal mucosa. In this regard, ganglioside GM1 decreased arachidonyl-specific PLA2 activity in neuroblastoma cells treated with ethanol (16). Gangliosides inhibit plasmalogen-selective PLA2 in bovine brain (15). The activity of PLA2 might be attenuated by cholesterol reduction (37,38) as our previous study demonstrated that dietary ganglioside decreased cholesterol content in the intestine (17). The present study demonstrates that dietary ganglioside has a therapeutic potential for the clinical use as an

inhibitor of PAF in acute gastrointestinal diseases. Arachidonic acid (20:4n-6) released by PLA2 is used for synthesis of eicosanoids, such as PGE2 and LTB4 by cyclooxygenase (COX) or lipoxygenase (LOX) (25). Several studies demonstrate that increased production of eicosanoids, especially LTB4, during inflammation or caused by endotoxin is dependent on PAF concentration (24,39,40). These inflammatory effects of PAF are blocked or attenuated by PAF antagonists (24,39-41). Inhibitors of COX or thromboxane synthetase were not able to reduce the damage induced by PAF while dual inhibitors of COX/LOX can reduce gastric damage (42). These results suggest a pivotal involvement of leukotrienes in PAF-induced intestinal mucosal damage. Thus, the effect of dietary ganglioside on inhibition of LTB4 and PGE2 secretion in both inflamed intestinal mucosa and blood by LPS indicates an anti-inflammatory effect of dietary ganglioside by an inhibition of PAF. This result is consistent with a previous study in our lab demonstrating that dietary ganglioside decreased PAF content in microdomains (17). It also suggests that dietary ganglioside may disrupt the localization of PAF or PAF receptors in microdomains where in many signaling molecules are enriched.

The role of LTB4 on IL-1β and TNF-α production is suggested in several studies (40,43). PAF induces high expression of both IL-1β and TNF-α cytokines upon inflammatory stimulation (26,44). These findings indicate inflammatory downstream targets of PAF into LTB4 leading to IL-1β and TNF-α stimulation because these inflammatory signals can be blocked by PAF antagonists (45-47) or PAF-acetylhydrolase (26,27). The present results thus suggest that dietary ganglioside-induced decreases in systematic circulating IL-1β and TNF-α content may be due to the inhibition of PAF production. As LTB4 recycles continuously from the intercellular to the extracellular membrane during inflammation, the recycling of LTB4 makes the inflammation worse by stimulating inflammatory cytokine and enzyme synthesis through activation of protein kinases and NF-κB (48). Increased ganglioside in the intestine also directly inhibits TNF secretion. Several gangliosides such as GD3, GD1a, GM3, GM2, and GM1 decreases LPS- or PAF-stimulated TNF production 5-fold in peripheral blood mononuclear cells (49). Several studies show inhibition of PKC activity by gangliosides (50,51). Further investigation is needed to determine whether dietary ganglioside-associated anti-inflammatory effects are related to inhibition of protein kinase cascade. Also it remains to

be demonstrated if dietary ganglioside can confer gut protection during prolonged exposure to LPS.

Taken together, the present data demonstrates that a physiological level of dietary ganglioside has significant anti-inflammatory effects by inhibiting PAF, PGE2, LTB4, IL-1β and TNF-α in gut inflamed by LPS. This study suggests a biological importance of gangliosides in gut development and a therapeutic potential for dietary gangliosides in acute gastrointestinal disease.

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CHAPTER IX
CONCLUSIONS AND DISCUSSION

9.1. OVERALL THESIS CONCLUSION

The hypotheses tested in the thesis have been addressed as follows:

Hypothesis 1: Dietary ganglioside will increase the content of gangliosides and alter the composition of gangliosides in the intestine, blood and brain in developing rats.

Dietary ganglioside significantly increased the total content of gangliosides in the intestine and in brain through the circulatory system during early development (Chapter III). The compositional change in gangliosides was observed only in the intestine with a decrease in GM3 and an increase in GD3. Although the dosage of sphingomyelin, a sphingolipid without sugars and N-acetyl sialic acids (NANA), was 10 times higher than that of ganglioside in the diet, neither the content nor the composition of gangliosides in tissues and blood tested was affected by the sphingomyelin diet.

This study suggests two major observations. First, since changes in the content and composition of ganglioside were altered by the GG diet and not the SM diet, NANA in gangliosides is likely an important component for the change occurring in the lipid profile. Secondly, the compositional change was tissue specific. This suggests that the brain has regulated mechanisms controlling the ganglioside composition in comparison to the intestine. This study also suggests that functional roles of gangliosides are different from those of sphingomyelin during early development.

Hypothesis 2: Intestinal gangliosides will have a specific localization at the intestinal membrane.

Fluorescent confocal microscopy showed that GM3 is almost exclusively localized at the brush border membrane (BBM) of the enterocyte while GD3 is situated at the basolateral membrane (BLM) with a minor localization at the BBM (Chapter III). The

selective localization of each ganglioside in the enterocyte suggests a specific function for each ganglioside due to intracellular location.

Hypothesis 3: Diet-induced increase in membrane gangliosides will change the ganglioside profile in photoreceptors of the developing retina.

The total content of ganglioside in the developing retina was significantly increased by dietary ganglioside (Chapter IV). Dietary ganglioside increased GD3, which is a biomarker of retinal differentiation and maturation in the developing retina. Unexpectedly, dietary LCP induced an increase in GD3 in the developing retina. This study suggests for the first time that the effect of PUFA on the improvement of visual acuity may occur through alteration of the ganglioside profile. Thus, it may indicate a new mechanism by which retinal function is influenced by these dietary lipids during development.

Hypothesis 4: Derivatives of dietary ganglioside will increase phospholipid content and decrease cholesterol content in the intestine.

Diet-induced increase of membrane gangliosides decreased the content of cholesterol in the intestine, brain and blood. (Chapter III). Animals fed the SM diet showed a cholesterol reduction in the intestine but not in the brain and blood. The degree of cholesterol reduction for the SM diet was similar to that of the GG diet.

Feeding animals the GG diet reduced total phospholipids in the intestine with a concomitant decrease in phosphatidylcholine (PC) (Chapter V). Animals fed the SM diet did not demonstrate a change in total phospholipids but showed increases in SM and phosphatidylethanolamine (PE) and a decrease in PC. Although the high dosage of SM in the diet was enough to alter the phospholipid composition, total phospholipid was not altered by the SM diet. This data suggests that NANA or sugar(s) in gangliosides may affect phospholipid synthesis or incorporation into the membrane during gut development.

Levels of PUFA, including 20:5n-3, were increased in phosphatidylserine (PS), phosphatidylinositol (PI), and PE. These results may have been caused by activation of Δ4/Δ5 desaturase or intestinal/liver fatty acid binding protein (FABP) by the GG diet-induced cholesterol reduction.

Hypothesis 5: Derivatives of dietary ganglioside will increase the synthesis of ether phospholipids in the intestine.

Animals fed dietary ganglioside exhibited a significant increase in both total content and molecular percentage of ether phospholipids in the choline phosphoglyceride (CPG) and ethanolamine phosphoglyceride (EPG) fraction in the intestine during development (Chapter VI). PUFA including 20:4n-6 and 22:6n-3 was highly incorporated into ether phospholipids, leading to a decrease in the ratio of saturated fatty acids (SFA) to PUFA both in CPG and in EPG. The effect on total ether phospholipids and PUFA by the SM diet was less pronounced than that of the GG diet. This study suggests that dietary sphingolipids increase ether phospholipid synthesis as well as PUFA in the intestine during development.

Hypothesis 6: Dietary ganglioside will increase ganglioside content and decrease cholesterol content in intestinal microdomains during development.

Hypothesis 7: Diet-induced cholesterol reduction in the intestine will decrease caveolin expression in intestinal microdomains.

Hypothesis 8: Decreased caveolin content in microdomains decreases pro-inflammatory mediators, PAF and DG in microdomains.

Hypotheses 6, 7 and 8 were addressed by Chapter VII. The effect of dietary ganglioside on the lipid profile of intestinal microdomains was similar to that observed in intestinal mucosa (Chapter III) during development. Animals fed dietary ganglioside showed significant increases in total gangliosides and sphingomyelin and a decrease in cholesterol content in microdomains. As a result, the ratio of cholesterol to gangliosides and cholesterol to sphingomyelin was reduced. Despite causing cholesterol reduction in

microdomains, the LCP diet did not change the sphingolipid content or the ratio of cholesterol to gangliosides and cholesterol to sphingomyelin. Diet-induced cholesterol reduction significantly decreased caveolin content in intestinal microdomains. This study suggests that cholesterol reduction may be an important factor to interrupt caveolin expression or localization in microdomains. Animals fed dietary ganglioside also showed reduced levels of platelet activating factor (PAF) and diglyceride (DG) in microdomains. These results may be due to a structural change in microdomains as a result of lower cholesterol and caveolin expression, perhaps leading to delocalization of PAF or its receptor and DG in microdomains. The GG diet was more effective than the LCP diet in reducing PAF and DG levels.

Hypothesis 9: Dietary ganglioside will inhibit pro-inflammatory signals, PAF, PGE2, LTB4, IL-1β and TNF-α in the intestine and blood inflamed by LPS exposure.

When animals were infected by LPS, animals fed the control diet showed a significant decrease in total gangliosides while the group fed the GG diet showed a significant increase in total gangliosides in the intestine (Chapter VIII). Animals fed the control diet showed a remarkable increase in PAF content in the intestine of LPS animals while dietary ganglioside caused a decrease in PAF production in both saline and LPS animals. Feeding animals the GG diet reduced PGE2 and LTB4 production in the intestine and decreased LTB4, IL-1β and TNF-α in blood of LPS animals. This study suggests that dietary ganglioside significantly inhibits inflammation or infection by decreasing pro-inflammatory signals in the intestine and blood after exposure to LPS.

9.2. OVERALL THESIS DISCUSSION

The present thesis demonstrates that dietary ganglioside (1) increases total gangliosides; (2) alters the composition of gangliosides; (3) decreases cholesterol content in the intestine, blood, retina and brain; (4) changes the phospholipid profile; (5) increases ether phospholipids; (6) enhances polyunsaturated fatty acid (PUFA); (7) decreases caveolin expression; and (8) inhibits inflammatory signals by LPS injection

during gut development. This thesis also (9) describes the localization of ganglioside GM3 and GD3 in the enterocyte.

This is the first research to demonstrate a selective localization of ganglioside in the enterocyte. This is significant because it highlights the functional roles of gangliosides. The location of GM3 on the apical membrane may facilitate its role as a first-line defence against bacterial invasion. The location of GD3 on the basolateral membrane may enhance its communication with immune cells in the lamina propria. The specific functions of these and other gangliosides need to be examined further in relation to their localization. The localization of gangliosides may provide valuable insights into their function and roles in gut physiology.

A new relationship between dietary LCP and gangliosides in the retina was discovered. The implication of this important finding is that LCP may have effects on visual function by altering the ganglioside profile, specifically increasing GD3 in the developing photoreceptor. This finding opens the door to new research areas in understanding and treating retinal pathological processes. Many retinal pathologies are associated with low retinal LCP. It would be of interest to elucidate the role of ganglioside in retinal disease.

This is the first study to demonstrate metabolic conversion of dietary sphingolipids to ether phospholipids in developing intestine. Since ether phospholipids have antioxidant activity, they may play a role in preventing cancer in tissues subjected to oxidative stress. With the exception of neuronal tissues, intestines have the highest levels of ether phospholipids of all tissues. High levels of ether phospholipids in the intestine may serve to protect gut membranes from peroxidation induced by free radicals associated with digesting food. Since humans or animals that suffer from certain neuronal diseases or disorders lack ether phospholipids such as Zellweger syndrome, Alzheimer's disease and Refsum disease, it may be of interest to examine the potential of dietary gangliosides for therapeutic roles.

For the first time, dietary ganglioside was shown to mitigate the effects of LPS toxin on the intestine. A direct implication of this finding is that dietary ganglioside may inhibit infant or childhood diarrhea caused by inflammation due to acute bacterial

infection. From a broader perspective, this research may be relevant to chronic inflammatory processes of the intestine, such as Crohn's disease or necrotizing colitis.

Unlike mothers' milk, most infant formulas currently commercially available contain little to no ganglioside. In light of the importance of dietary ganglioside during developmental periods in this rat model, ganglioside supplementation during these critical times should be considered for human infants. The importance of dietary ganglioside needs to be examined through human clinical studies. Since relatively little is known about the relationship between nutrition and physiology of gangliosides, nutritional ganglioside research offers the potential of significant gains in knowledge.

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